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Morphometric, Isozyme and Random Amplified Polymorphic DNA (RAPD) : Analysis of SAROTHERODON MOSSAMBICUS Populations in Java, Indonesia

Yoedono Sovyanhadi

ABSTRACT

MORPHOMETRIC, ISOZYME AND RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS OF Sarotherodon mossambicus POPULATIONS IN JAVA, INDONESIA

by

Yoedono Sovyanhadi

The tilapia fish Sarotherodon mossambicus (Teleostei, Cichlidae) is an important protein source for Indonesians, is a strong competitor with some endemic species and readily hybridizes with other tilapias. A base line assessment of the present genetic diversity of these populations in Indonesia was conducted to facilitate the management of this species. This study compared the effectiveness of the 3 techniques (morphometric, isozyme and RAPD) in revealing differences within and among 9 Javanese populations. The data were also used to estimate the rate of divergence among the populations.

Morphometric analysis showed significant size and shape differences among the populations. Isozyme and RAPD analysis also showed significant population differentiation (G_{st} = 0.067; χ^2= 30.15 ; $p<0.01$ and G_{st} = 0.217; χ^2 = 38.95; $p<0.01$ and average genetic distance D of 0.005 and 0.136,

respectively). Morphological differences appear to be primarily environmentally induced, since morphological data were inconsistent with molecular genetic data. The putative date of the introduction of this species to Java along with population diversity measures indicate rapid genetic change, possibly the result of an extreme founder effect.

LOMA LINDA UNIVERSITY

Graduate School

MORPHOMETRIC, ISOZYME AND RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS OF *Sarotherodon mossambicus* POPULATIONS IN JAVA, INDONESIA

by

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Each person whose signature appears below certifies that this dissertation, in his opinion, is adequate in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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CHAPTER ONE

GENERAL INTRODUCTION

Genetic variations found in natural populations represent fundamental resources needed for present and future survival of any species. These variations potentiate populations for adaptive evolution as well as for speciation. Therefore it is essential to conserve the wealth of genetic resources for future generations (Lannan et al., 1989).

Genetic variation in a population can be depleted by population bottle necking, inbreeding, intense selection or by hybridization leading to reticulation. These causes of genetic deterioration can be represented by the following 3 types of production systems in fisheries (Lannan et al., 1989): a) 'capture fisheries' (harvesting of naturally reproducing wild populations of fish): this practice has at times led to a rapid decrease in fish density resulting in genetic homogeneity of the population due to drift and/or inbreeding; b) 'aquaculture' (farming or ranching of aquatic species): this practice places emphasis on the immediate improvement of production traits through artificial selection, resulting in drastic reduction of genetic

diversity of broodstocks; c) 'culture-based fisheries' (employing aquaculture to produce juveniles for capture fisheries): this practice commonly performs uncontrolled intra/interspecific hybridizations in hatcheries, resulting in extensive genetic mixture of the progenies and thus replacing the parental genetic variation. Pullin (1988) presented the case of the disappearance of the native Sarotherodon aureus stock in the Jordan Valley, Israel as the consequence of extreme loss of parental genetic variation. This species was found as a well isolated population in the northwest shore of the Dead Sea in 1964. Since the 1970s, this native stock of S. aureus has mixed with S. niloticus which escaped from fish farms in Upper Galilee. Moreover, the open waters such as Lake Kinneret are stocked with fingerlings of 'niloticus-aureus' hybrids every year. Consequently, the native pure line S. aureus has been eliminated from that area by hybrid competition and introgressive hybridization.

Proper management of natural fish populations is necessary in order to sustain populations for harvests to meet fishery needs. Fishery management has been defined as the application of scientific knowledge to the problems of providing the optimum yield of commercial fisheries products or angling pleasure (Everhart and Youngs, 1981). The

objective of such management is to maximize the harvest while conserving the resource. According to Allendorf et al. (1987), fishery management has largely been concerned with the abundance and size of fish available for harvesting simply by selecting an appropriate balance between harvest and recruitment. Fishery management plans must move beyond such short term goals and in addition address issues of differential survival and reproduction of individuals with different genotypes. In the long view, fishery management should be the conservation of existing resources to ensure a sustainable yield for the future. Therefore, management should be aimed at maintaining genetic variation within populations, since the genetic resources represent the potential for present and future harvests of the fish. Genetic variation in natural populations also represent the germ plasm for present and future development of improved fish aquaculture varieties.

An example of the importance of such fishery management is given by Hynes et al. (1981) and Pullin (1988) when they discussed ways to maintain genetic diversity in the broodstock populations. Ideally, at the beginning of a selection program broodstocks should be taken from a broad genetic base of the naturally reproducing wild populations. Such practice usually results in the immediate improvement

of production traits such as growth, disease resistance, food conversion rate and flesh quality. In practice, however, the broodstocks are taken from a limited number of individuals due to economic reasons. Consequently, the genetic variation in the broodstock population is limited and the population very soon becomes genetically poor and a small subset of the genetic diversity represented in the ancestral populations. In order to prevent the elimination of the broodstock population as well as the cultured stock that has genetically deteriorated, individuals from natural populations should be included into the breeding stocks to provide additional genetic diversity. Conservation of the genetic resources through proper management of natural fish populations is therefore also essential for aquaculture.

A first step in the management of a fish population is to document the genetic resources of the population. Resulting documents serve as the basic data for the development of management policies of those populations. McAndrew and Majumdar (1983) give examples of management decisions to be made once the documentation of the available species is confirmed. If the stock is still pure, every effort should be made to ensure that it remains undisturbed. If the stock is a hybridized population, the decision to start with new pure species may be taken; alternatively,

selection of a strain to suit the local conditions can be done based on the genetic variation in that stock.

Documentation procedures to identify populations and their genetic variations can be performed at the morphological, protein and DNA levels of analysis. Morphometric and meristic data are commonly used as the measure of morphological variation among fish populations (Khater, 1985). At first, these data could only distinguish interspecific differences due to the great variabilities within the species. However, refined morphological techniques have been developed for detecting intraspecific variations. Pante (1988) used canonical discriminant analysis of morphometric and meristic data to clearly separate the populations of O. mosambicus from O. niloticus. Brzeski and Doyle (1988), using only morphometric data obtained from the truss network technique (Strauss and Bookstein, 1982), were able to determine the sex of tilapia. Another multivariate morphometric approach was developed to allow comparison of allometric growth of different fishes while removing the effect of size from shape differences (Humphries et al., 1981; Bookstein et al., 1985). This technique, known as sheared Principle Component Analysis, has been used to demonstrate intraspecific morphometric

variabilities among fish populations (Winan, 1984; Warren, 1992; Marais, 1993).

At the protein level, enzyme electrophoresis is a common tool for documenting the genetic status of wild and cultured fish populations. This technique can provide genetic markers and estimate the heterozygosities and genetic distances. Several studies have shown protein electrophoresis to be useful for species identification in fishes (Cruz et al., 1982; McAndrew and Majumdar, 1983; Verheyen et al., 1985; Brummet et al., 1988a and b). Protein electrophoresis has also been used in many population studies for investigating intraspecific variations in fishes (Crozier and Ferguson, 1986; Stahl, 1987; Gyllensten and Ryman, 1988; De Silva and Ranasinghe, 1989; Hedgecock and Sly, 1990; Sanchez et al., 1991; Gaffney et al., 1992).

DNA analysis can be an even more powerful tool for resolving intraspecific differences. DNA hybridization with specific mitochondrial-DNA and ribosomal-DNA probes as well as analysis of specific DNA products produced by the polymerase chain reaction (PCR) can provide valuable insight into molecular events and differences within fish populations (Crozier and Ferguson, 1986; Gonzalez-Villasenor et al., 1986; Wright, 1989; Gonzalez-Villasenor and Powers,

1990). When combined with protein studies, the DNA electrophoresis may even delineate speciation mechanisms (Ovenden and White, 1990). Harris et al. (1991) showed that DNA fingerprints can be produced for identifying individuals and family groups, and for broodstock labelling to secure ownership. A simple procedure using random amplified polymorphic DNA (RAPD) markers has also been useful for revealing inter- as well as intraspecific genetic differences among fish populations (Dinesh et al., 1993; Johnson et al., 1994; Postlethwait et al., 1994; Bardakci and Skibinski, 1994).

In this study, documentation at the morphological level (morphometric data), protein level (isozyme data) and DNA level (RAPD data) was done to compare the effectiveness of each technique in revealing the differences among S. mossambicus populations in Java. Such information provides aquaculturists with reproducible and efficient morphometric and molecular techniques optimized for tilapia identification. The data will also serve as a baseline analysis of the current genetic diversity found among introduced S. mossambicus populations living freely in Javanese water systems.

Tilapia aquaculture farms are numerous particularly in West Java and are sources of several tilapia species and

hybrids that may have been introduced purposely or by accident into the surrounding natural waters. Since hybridization among tilapia species is quite common (Chen, 1988), individuals that escape from farms readily mate with S. mossambicus in natural waters. Such hybridization when uncontrolled could eventually eliminate the pure strain of S. mossambicus from natural waters. The genetic resources of tilapia populations in Java, Indonesia has never been documented. The fact that S. mossambicus is an important protein source for human consumption that has not yet been fully exploited provides a timely opportunity to make fundamental genetic assessments before further population divergence can be affected by tilapia farming. The data provide information for management of S. mossambicus stocks in Java, particularly as it relates to the conservation of natural genetic diversity.

Besides their significance for fisheries management, the population genetic data obtained in this study may also have implications for reconstructing the history of the populations, especially the possible results of an extreme founder effect. According to Pullin (1988), the present populations of S. mossambicus in Java may descend from 2 females and 3 males introduced in 1939. There is no concrete data about how the fishes were distributed

throughout the island. However, Vaas and Hofstede (1952) believed that each population was started from some founders through local transfer. Extreme founder event such as that proposed by Pullin (1988) should decrease the percent polymorphism and result in population differentiation (Hartl and Clark, 1989).

CHAPTER TWO

MORPHOMETRIC STUDY OF S. mossambicus POPULATIONS IN JAVA

INTRODUCTION

The causes and limits to biological diversity are one of the central themes studied by biologists. Morphological characters have been used as the major criterion for classifying and differentiating groups of organisms throughout the history of taxonomy (Mayr and Ashlock, 1991). The study of morphology has indeed provided the basic insights into classification and structure-function relationships for systematics.

Lele and Richtsmeier (1990) and Rohlf (1990) defined morphometrics as the quantitative analysis, description and interpretation of size and shape variation in biology. According to Rohlf and Bookstein (1990), all morphometric implementations within a multivariate statistical analysis are governed by the concept of structural homology. Homology is a matter of correspondence between biological parts which are similar in fundamental structure, position or development. This traditional idea also applies to the New Systematics view of relatedness. The homologous parts

are represented by variables such as physical distances, net areas or volumes, angles of articulation between substructures, and ratios among quantities. In the classical multivariate morphometrics, however, the concept of homology was present only implicitly. There was no formal criterion by which the concept of homology could be transferred from a biological domain into a statistical framework. Therefore, the interpretation of the results were often biased and the geometry of the objects was discarded because it could not be recovered after the analysis was completed.

An extension of the notion of homology into biometrics was pioneered by Thomson (1961). In its revised version, the concept of homology is considered as a mapping function, a correspondence relating points to points rather than parts to parts. The data consist of measurements of discrete points or 'landmarks' which correspond among all the forms. These landmark data therefore simultaneously contain information about geometry, homology, and can be evaluated using multivariate statistics. This morphometric technique was made known in 1971 when Blackith and Reyment published their book entitled 'Multivariate Morphometrics'.

With the development of the scientific theory of classification, modification of morphometric techniques for

systematic purposes became the main interest of taxonomic study. Biological morphometrics which is usually termed as numerical taxonomy or phenetics, therefore, underwent changes in order to relate organisms objectively in terms of their overall similarity, assessed by measurements of body shape and other quantitative traits (Strauss, 1991).

Changes in the study of biological morphometrics in the past ten years have occurred primarily to deal with geometric aspects of shape and the allometric models of growth and size variation. The geometric perspective on shape graphically depicts the point-by-point geometric transformation of one form to another, whereas allometric models are necessary for biological comparisons because they furnish a firm biological basis for data interpretation.

A powerful methodology for exploring the diversity of body shape was then developed that combined the quantification of shape with confirmatory multivariate statistics (Strauss, 1991). The new morphometric technique requires adequate information about the shape of the structure so that the shape can be reconstructed from those data. At the same time, selection of efficient variables is necessary in order to limit the volume of the data (Rohlf, 1990). Two kinds of data, called distances and coordinates, can be generated from landmark data to represent the form of

an object. Distance data are quantitative descriptions of the length of an object or a measure of separation between 2 parts of an organism. In contrast, coordinate data represent points in a grid which are described by an 'x' and a 'y'. Outline data, another type of measurement, represents the summations of coordinate data around the periphery of a form.

One approach used in the collection of distance measures from landmark points is the even distribution of measures over a shape in a pattern of quadrilaterals called 'box trusses'. In this way, a set of landmark locations is divided into neighborhoods of 4 points each, then each neighborhood is measured exhaustively by all 6 interpoint distances. This approach is preferred for 3 reasons: a) the pattern systematically detects shape differences in oblique as well as horizontal and vertical direction; b) the configuration of landmarks is preserved so that the shape can be reconstructed; and c) random measurement errors are recognized, but compensated for by modest and systematically redundant distance measurements.

Analysis of the data is objectively conducted so that group discrimination and/or description of shape change are derived as the products of the analysis without prior selection of favored characters (Humphries et al, 1981;

Bookstein et al, 1985). Distance data are suitable for analysis using multivariate methods such as Principal Component Analysis (PCA), Principal Coordinate Analysis (PCO) and Discriminant Function Analysis (DFA). Principal Component Analysis (PCA) and PCO identify the few factors that account for most of the variation; PCA is based on the correlations or covariances among the observed variables without prior knowledge about the groups of the sample. PCO can be performed using any kind of distance matrix among the sample groups. DFA is used to find a few indices or 'canonical functions' in the form of linear combinations of the observed variables, that best discriminate the already known sample groups. To identify the shapes of groups that differ in size, a sheared-principal component analysis (sheared-PCA) is used. Sheared-PCA treats both size and shape as unmeasured factors, then the size factor is sheared leaving the shape component alone. Therefore, sheared-PCA can show the shape variation among the sample groups with a minimum size effect.

Box truss data analyzed by sheared-PCA have been particularly important in studies of fish. Humphries et al (1981), using box truss and sheared-PCA analysis, have successfully identified shape differences between two populations of Cyprinodon and among populations of

Rhinichthys and Richardsonius. Strauss and Bookstein (1982), using sheared-PCA, compared the box truss with the traditional morphometric measures. They were able to obtain a total separation in shape of two population of Cottus with the box truss only. Box truss and/or sheared-PCA were also used in distinguishing stocks of various marine fish species such as Chinook salmon (Winan, 1984) and pollock and haddock (McGlade and Boulding, 1985); in the study of growth patterns and sex differentiation of various age groups in tilapia (Brzeski and Doyle, 1988); in the study of the effects of recovery efforts on populations of the ciprinid Gila purpurea (Warren, 1992); and were successfully used to cluster 27 populations of Lepomis into six geographic regions (Marais, 1993).

The purposes of this morphometric study were as follows: 1) to document size and shape variation among S. mossambicus populations in Java; 2) to determine the effectiveness of morphometric data in discriminating among populations; and 3) to assess the effectiveness of morphometric data for documenting genetic diversity.

MATERIALS AND METHODS

Sample Collection and Treatment

Thirty Javanese tilapia (S. mossambicus) were randomly collected from each of 9 isolated sites along the island of Java (Table 1; Figure 1). Three sites each were selected from East, Central and West Java. The main consideration in selecting the sites was geographic separation from each other. The selected sites were on different tributaries and relatively far from each other (about 200 kilometers apart), so that contact among populations by natural migration should be unlikely to occur. These sites also represented diverse environmental conditions in terms of the size of the water body, water salinity and temperature, altitude and tidal fluctuation (Table 1).

Specimens were caught using a throw-net and killed in ice, then preserved for morphometric study following the procedure of Khater (1985): soaked in 10% formalin for 24 hours; drained; rinsed and soaked in water for 24 hours; dehydrated in alcohol for 3 days; alcohol replaced with fresh absolute alcohol as preservative.

Table 1. Sites of the Populations of S. mossambicus Under Study

Pop	Site	Altitude; Temp. (m) ; (C°)	Area ; Depth (m^2) ; (m)	Type of Water Body
W-1	Serang West Java	± 10 ; > 25	< 15,000 ; 1.25	less fluctuating tidal swamp; near community housing
W-2	Situ Bagendit West Java	± 1,000 ; < 20	>10';deep water	freshwater lake; stable conditions
W-3	Losari West Java	± 10 ; > 25	± 10,000 ; 0.75	less fluctuating tidal swamp; near commonity housing
C-1	Pekalongan Central Java	± 10 ; > 25	< 15,000 ; 1.25	less fluctuating tidal swamp; near community housing
C-2	Magelang Central Java	±400 ; > 20	< 10,000 ; 0.75	freshwater spring; near community housing; water level about stable
C-3	Rembang Central Java	± 10 ; > 25	< 10,000 ; 0.75	open tidal swamp;fluctuating water level
E-1	Sarangan East Java	1,200 ; < 20	>10';deep water	freshwater lake; stable conditions
E-2	Sidoarjo East Java	± 10 ; > 25	< 10,000 ; 0.75	tidal swamp; near crowded city; near community housing
E-3	Situbondo East Java	± 10 ; > 25	± 25,000 ; 0.75	tidal swamp; near community housing; water level fairly stable

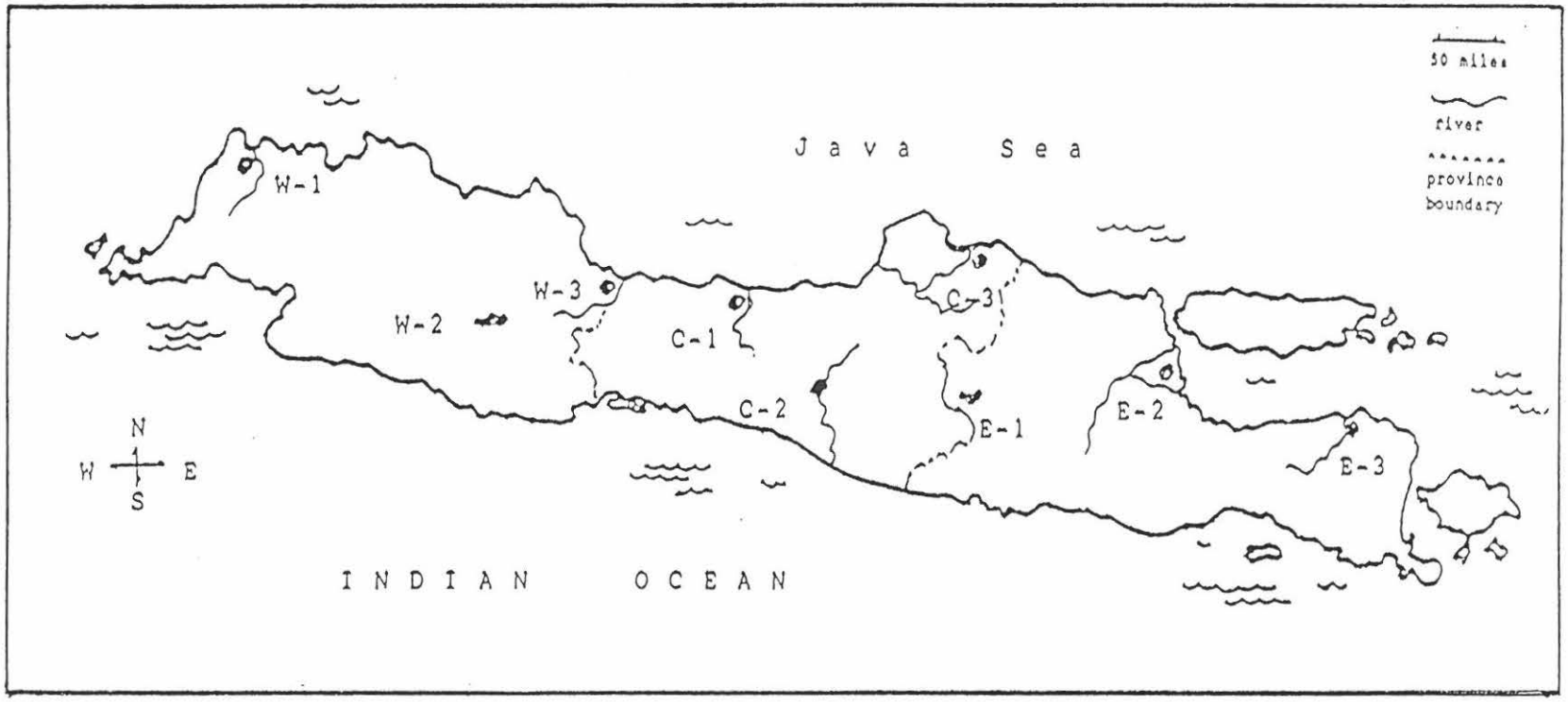

Figure 1. Map of Java Showing the 9 Sampling Sites: 1) W-1: Serang-West Java; 2) W-2: Situ Bagendit-West Java; 3) W-3: Losari-West Java; 4) C-1: Pekalongan-Central Java; 5) C-2: Magelang-Central Java; 6) C-3: Rembang-Central Java; 7) E-1: Tlaga Wurung-East Java; 8) E-2: Sidoarjo-East Java; 9) E-3: Situbondo-East Java. Thirty individuals per site were used for analysis in this study.

Data Collection

The morphometric measurements were collected according to the truss technique in which the framework of body form is measured (Strauss and Bookstein, 1982; Brzeski and Doyle, 1988). Ten distinctive and homologous landmarks on the outline of the fish were first selected to represent the body form (Figure 2). Each landmark denotes a certain point within the body frame of an individual fish which is geometrically homologous across all samples. The ten landmarks of an individual were then mathematically connected to each other to generate a series of contiguous quadrilaterals, each having two internal diagonals. Each quadrilateral thus shared one edge with the preceding quadrilateral and another edge with the succeeding quadrilateral. The measured distances between landmarks served as variables for morphometric analysis. This truss network can therefore provide 21 different variables (Table 2).

In order to obtain a planar image of the fish, each sample was photocopied in a standard manner. The two dimensional photocopied image provided 21 landmark distances that were measured using a digital caliper with a precision of 0.01 mm.

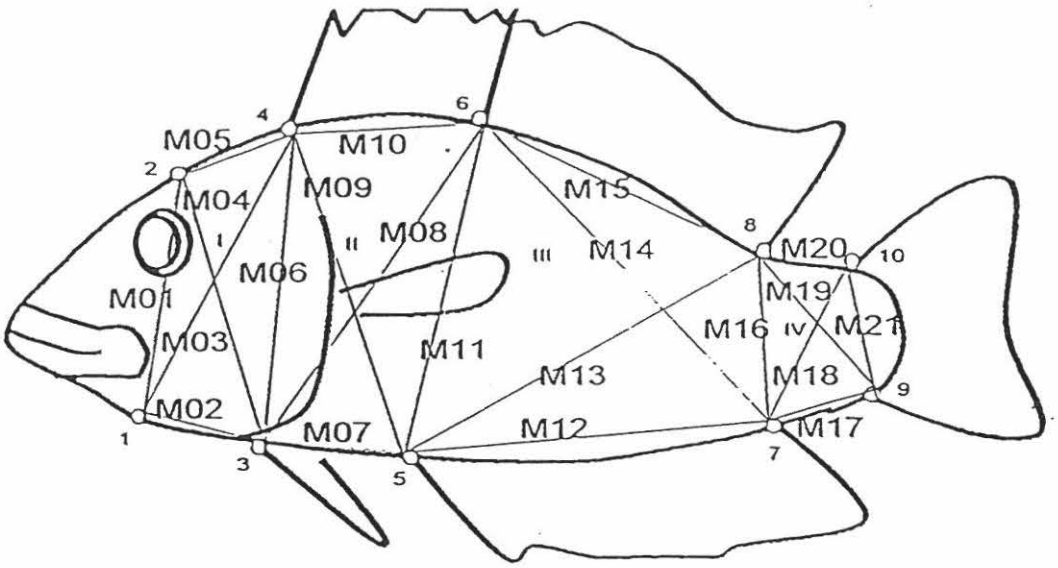

Figure 2. Landmarks and Truss Distances:
1) posteriormost point of maxilla; 2) posteriormost point of eye; 3) origin of pelvic fin; 4) origin of dorsal fin; 5) origin of anal fin; 6) point between the spinous and soft portions of dorsal fin; 7) the insertion of anal fin; 8) the insertion of dorsal fin; 9) anterior attachment of the ventral membrane of caudal fin; 10) anterior attachment of the dorsal membrane of caudal fin; I to IV are the quadrilaterals; M01 to M21 are the distances that become the variables in the analysis.

Table 2. The 21 Variables of the Truss Morphometrics

Sequence Distance	Quadrilateral	Variable
Point 1 to 2	I	M01
Point 1 to 3	I	M02
Point 1 to 4	I	M03
Point 2 to 3	I	M04
Point 2 to 4	I	M05
Point 3 to 4	II	M06
Point 3 to 5	II	M07
Point 3 to 6	II	M08
Point 4 to 5	II	M09
Point 4 to 6	II	M10
Point 5 to 6	III	M11
Point 5 to 7	III	M12
Point 5 to 8	III	M13
Point 6 to 7	III	M14
Point 6 to 8	III	M15
Point 7 to 8	IV	M16
Point 7 to 9	IV	M17
Point 7 to 10	IV	M18
Point 8 to 9	IV	M19
Point 8 to 10	IV	M20
Point 9 to 10	IV	M21

Data Analysis

All truss lengths from the sampled fish (270 individuals) were entered into the computer and transformed into common logarithms. Principal Component Analysis (PCA) and Discriminant Function Analysis (DFA) were then used to analyze the log-transformed data set. The PCA and DFA were computed using microcomputer versions of SPSS and SYSTAT Windows Version 5.0 programs.

PCA was used to obtain a few uncorrelated, transformed variables (principal components or PC's) from the many original variables which were highly correlated with one another. The first principal component (PC-1), which typically represents size in morphometric studies, is uncorrelated with the subsequent principal components used in the analysis across the entire sample. The second principal component (PC-2) and the third principal component (PC-3) are expected to represent shape independent of size. However, size may still be carried within each of these principal components for subsets of the entire sample. Thus, for example, a PCA performed on a sample including subsamples of several species will have independent PC-1 and PC-2 across the entire sample. However, PC-1 (size) and PC-2 (shape) may be correlated within the subsample representing each species. To remove such correlations

between size and shape, and allow investigation of shape independent of size, the sheared-PCA technique was employed, following Humphries et al (1981) and Bookstein et al (1985). The conceptual steps of this shearing procedure are presented in Appendix 1.

In this study, PCA was conducted on a covariance matrix, since the variables, i.e. the 21 truss variables (Table 2), were measured in the same units. Each principal component (PC) is a linear combination of the 21 truss variables, and therefore 21 PC's were generated from the analysis. The first PC or PC-1 contributes the most to the total variation, PC-2 the second, and so on; all PC's cumulatively make up 100% of the total variation.

One-way analysis of variance (ANOVA) was performed on each retained principal component to test for differences among fish populations. As there were significant differences in the populations, the Scheffé post-test was performed to reveal the pair-wise differences among the fish populations.

DFA was used to find variables that contribute the most in the separation of the populations and to detect the maximum amount of multivariate variation across population means relative to within-population variation. This analysis also gives an assessment of how well the

morphological data can discriminate among the populations, and at the same time indicates the variables that contribute the most to the total variation among populations.

The average size-corrected shape of each population was estimated following the method of Strauss and Bookstein (1982) so that the shape differences among populations can be visualized. This method along with the calculation of individual composite size Sc is given in Appendix 2.

Truss lengths were transformed into logarithmic form (Bookstein et al, 1985; Marcus, 1990) in order to reduce the correlation between means and variances of the variables as well as the heterogenity of the variances because of differences in the magnitude of different variables.

RESULTS

The first 3 PC's from PCA were retained for further analysis since they accounted for 88.38% of the original variation. The loadings (correlation) of original variables with the first three principal components are shown in Table 3. All 21 variables loaded positively on PC-1 with scores ranging from 0.058 to 0.086. Because of the unipolarity and similarity in magnitude of these loadings,

Table 3. The Component Loadings of PC-1, PC-2, PC-3 and H *)

Variable	PC-1	PC-2	PC-3	H
M01	0.069606	0.004183	0.016322	0.004372
M02	0.074000	0.033708	0.016320	0.033912
M03	0.066710	0.005956	0.002812	0.006137
M04	0.076301	0.010420	0.008251	0.010628
M05	0.061481	0.009961	-0.033420	0.010128
M06	0.077162	0.002987	0.007023	0.003196
M07	0.063452	-0.007550	0.007023	-0.007379
M08	0.077353	-0.001797	0.008294	-0.007379
M09	0.075022	0.000689	0.008231	0.000892
M10	0.085059	0.007728	0.011053	0.007959
M11	0.081342	-0.007674	0.000762	-0.007455
M12	0.086372	-0.013500	0.000358	-0.013268
M13	0.082133	-0.013110	0.002408	-0.012889
M14	0.075328	-0.020530	-0.004733	-0.020328
M15	0.070530	-0.031870	-0.009122	-0.031683
M16	0.073038	-0.004304	-0.002800	-0.005107
M17	0.057500	0.014784	-0.014690	0.014941
M18	0.068346	-0.000882	-0.003092	-0.000697
M19	0.063646	0.005321	-0.011350	0.005494
M20	0.061876	0.023238	-0.022540	0.023408
M21	0.071460	-0.006005	-0.001972	-0.005812

note: *) 'Loadings' = correlation coefficients between each variable and PC-scores; 'Component loadings' = the covariances of the original variables with the PC-scores.

PC-1 was interpreted as a general size factor because the score of this PC-1 becomes larger with a larger value of any variable (Humphries et al., 1981; Meyer, 1990; Rohlf and Bookstein, 1990). This size factor accounted for 82.93% of the total variance. The similarity in magnitude of the loadings also implied that all of the variables were size indicators and that PC-1 contained within-population as well as between-population size differences (Humphries et al., 1981; Bookstein et al., 1985; Meyer, 1990).

That PC-1 expressed almost exclusively size is evident when PC-1 is plotted against the composite size Sc. Figure 3 and Table 4 show a high correlation between PC-1 and Sc ($r = 0.999$), and the strongly linear distribution of the samples indicates that PC-1 does represent a size factor. Figure 4 is a box plot of PC-1 by population and implies the existence of size variation within and between populations. ANOVA on PC-1 (Table 5a) demonstrates highly significant size differences among the 9 populations ($F = 25.8$; $p < 0.01$). Results of Scheffé's post-test are given in Table 5b. When the recorded environmental factors are qualitatively categorized (Table 5c), correlations between size and individual as well as combined environmental factors are not significant (Table 5d).

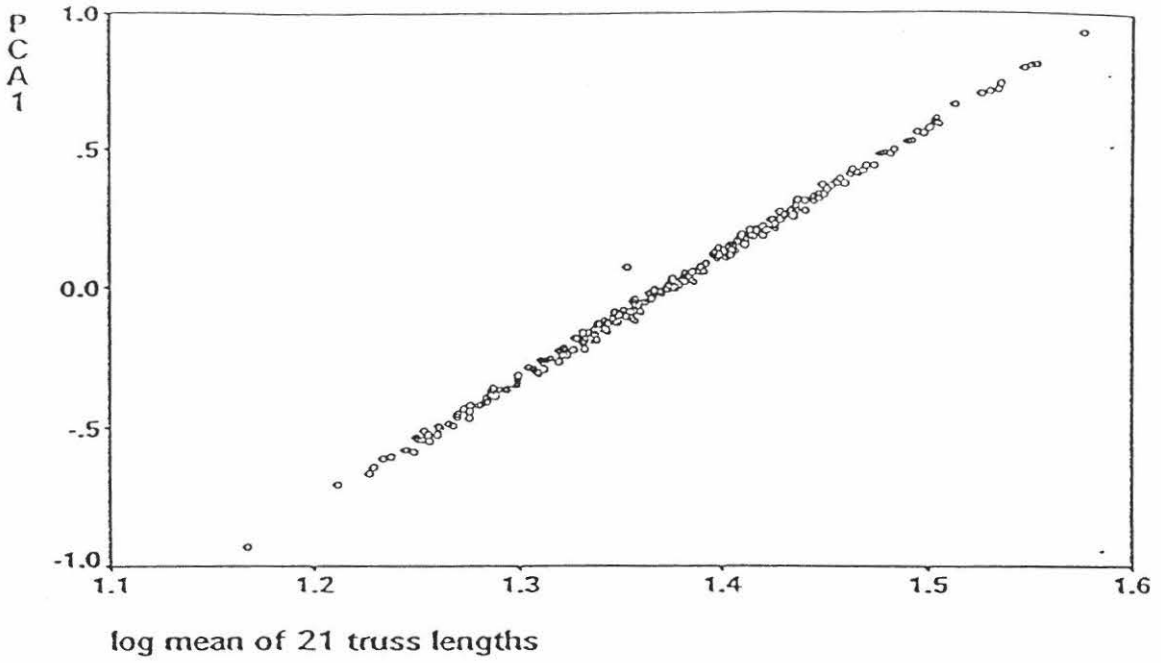

Figure 3. Relationship Between PCA-1 (PC-1) and Log Mean of 21 Truss Lengths (the Composite Size S_c).

Table 4. Pierson Correlations (= r) Between Various Variables

Variables	r	p-value	Variables	r	p-value
PC-1 vs Sc	0.999	0.000	S vs H	0.008	0.891
PC-1 vs H	0.015	0.811	S vs H'	-0.020	0.743
PC-1 vs H'	0.064	0.294	S vs PC-3	-0.108	0.076
$PC\text{-}1_{(total)}$ vs S	0.647	0.000	$PC\text{-}2_{(total)}$ vs S	-0.001	0.993
$PC\text{-}1_{(W\text{-}1)}$ vs S	0.980	0.000	$PC\text{-}2_{(W\text{-}1)}$ vs S	-0.170	0.370
$PC\text{-}1_{(W\text{-}2)}$ vs S	0.987	0.000	$PC\text{-}2_{(W\text{-}2)}$ vs S	-0.047	0.805
$PC\text{-}1_{(W\text{-}3)}$ vs S	0.995	0.000	$PC\text{-}2_{(W\text{-}3)}$ vs S	-0.293	0.116
$PC\text{-}1_{(C\text{-}1)}$ vs S	0.997	0.000	$PC\text{-}2_{(C\text{-}1)}$ vs S	0.139	0.464
$PC\text{-}1_{(C\text{-}2)}$ vs S	0.981	0.000	$PC\text{-}2_{(C\text{-}2)}$ vs S	-0.011	0.954
$PC\text{-}1_{(C\text{-}3)}$ vs S	0.987	0.000	$PC\text{-}2_{(C\text{-}3)}$ vs S	-0.099	0.603
$PC\text{-}1_{(E\text{-}1)}$ vs S	0.997	0.000	$PC\text{-}2_{(E\text{-}1)}$ vs S	0.228	0.226
$PC\text{-}1_{(E\text{-}2)}$ vs S	0.989	0.000	$PC\text{-}2_{(E\text{-}2)}$ vs S	-0.164	0.386
$PC\text{-}1_{(E\text{-}3)}$ vs S	0.989	0.000	$PC\text{-}2_{(E\text{-}3)}$ vs S	-0.028	0.882

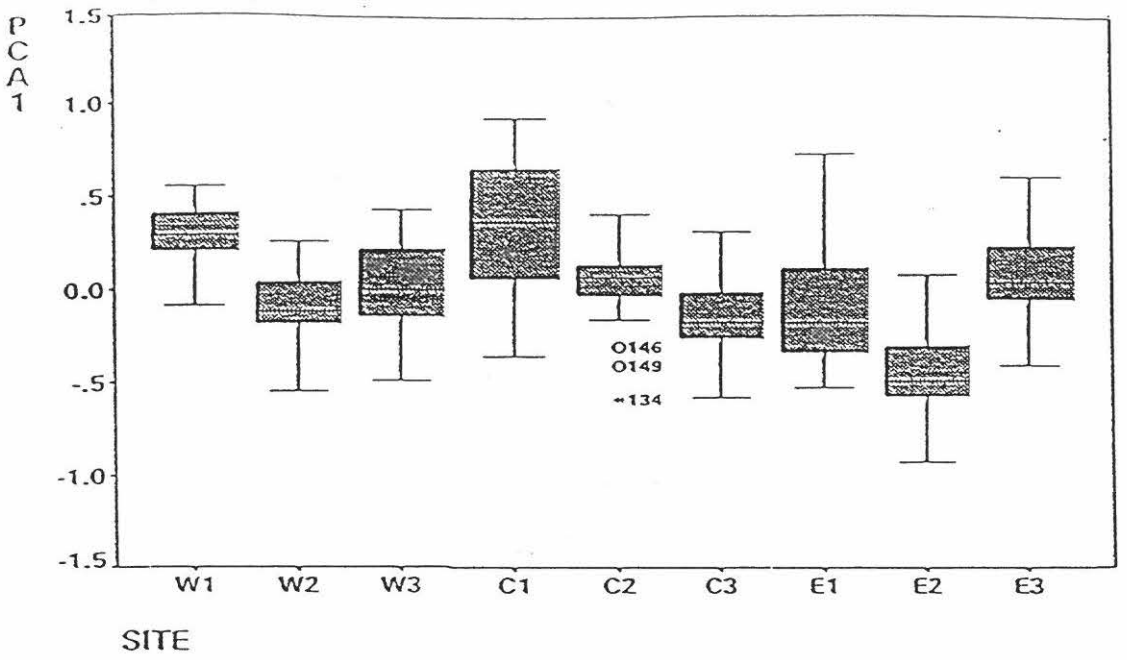

Figure 4. Box Plot of PCA-1 (PC-1) by Population Site. Circles and asterisk denote outliers.

Table 5a. Oneway Anova for PC-1 by Population

Source	df	SS	MS	F	P
Between Populations	8	13.183	1.6479	25.798	0.000
Within Populations	261	16.672	0.0639		
Total	269	29.855			

Table 5b. Scheffé Post-test for PC-1 by Population [1]

Pop.	Population								
	E-2	C-3	W-2	E-1	W-3	C-2	E-3	W-1	C-1
E-2									
C-3	*								
W-2	*								
E-1	*								
W-3	*								
C-2	*								
E-3	*								
W-1	*	*	*	*	*	*			
C-1	*	*	*	*	*	*			

note: 1) * means significant at 0.05 level

Table 5c. Recorded Environmental Conditions of the Sampling Sites. Type of Water Body and the Combined Factors are Qualitatively Categorized *)

Pop.	S-1	S-2	F-1	F-2	F-3	F-4	F-5	Combined Factors		
								F1;2	F3;4	All
W-1	4	3	10	25	15	1.25	3	1	2	4
W-2	2	3	1000	20	1000	250	4	3	4	7
W-3	2	1	10	25	10	0.75	3	1	1	3
C-1	4	3	10	25	15	1.25	3	1	2	4
C-2	2	5	400	22	10	0.75	3	2	1	6
C-3	2	2	10	25	10	0.75	2	1	1	2
E-1	2	5	1200	20	1000	500	4	4	5	8
E-2	1	3	10	25	10	0.75	1	1	1	1
E-3	3	4	10	25	25	0.75	3	1	3	5

*) S-1 = size; S-2 = shape; F-1 = altitude (m);
F-2 = temperature (C°); F-3 = area (m^2); F-4 = water depth (m)
F-5 = type of water body: 1: very unstable; 2: open, fluctuating water; 3: fairly stable, near housing; 4: lake, stable
F1;2 = combined altitude and temperature: 1: lower altitude, higher temperature; 4: higher altitude, lower temperature
F3;4 = combined area and depth: 1: smaller area and depth; 5: greater area and depth
All = combined all factors: 1: lower altitude, higher temperature, smaller area and depth, very unstable; 8: higher altitude, lower temperature, greater area and depth, stable water

Table 5d. Correlations Between Morphology (Size and Shape) and Environmental Factors

Variable	r	p -value
Size vs Altitude	-0.296	0.440
Size vs Temperature	0.318	0.405
Size vs Area	-0.243	0.529
Size vs Water depth	-0.231	0.549
Size vs Type of Waters	0.325	0.394
Size vs Altitude/Temp.	-0.294	0.442
Size vs Area/Water depth	0.093	0.813
Size vs All Factors	0.119	0.760
Shape vs Altitude	0.522	0.149
Shape vs Temperature	-0.562	0.116
Shape vs Area	0.342	0.368
Shape vs Water depth	0.453	0.221
Shape vs Type of Waters	0.333	0.381
Shape vs Altitude/Temp.	0.573	0.107
Shape vs Area/Water depth	0.490	0.181
Shape vs All Factors	0.673	0.047*

note: * is significant at 0.05 level

The within-population size component S is generated from the shearing of PC-2 and PC-3 (Appendix 1). The scatter plots between PC-1 (within and between-population size) against S (within-population size) reveal the between-population size, i.e. the size differences among populations, because the within-population size is made uniform across the populations (Figure 5).

The scatter plot of within-population size S against PC-2 suggested no evidence of positive correlation within any population sample (Figure 6). Thus, shearing of PC-2 was not required to remove any within-population size variance. PC-2 shearing was performed since sheared-PCA is considered the standard method of analyzing shape in studies of fishes (Humphries et al., 1981; Strauss and Bookstein, 1982; Bookstein et al., 1985). The sheared PC-2, H, likewise showed no correlation with PC-1 (Table 4).

The general shape of each individual was inferred from its H-score. Loadings on H (Table 3) contrast the variables M02, M04, M05, M17 and M20, which are positive, with M12, M13, M14 and M15, which are negative. Figure 7 shows the shape indicator variables M02, M04, M05, M12, M13, M14, M15, M17 and M20 that have high loadings on H. Positive and negative signs indicate the positive and negative loading scores of the corresponding variables. High positive mean

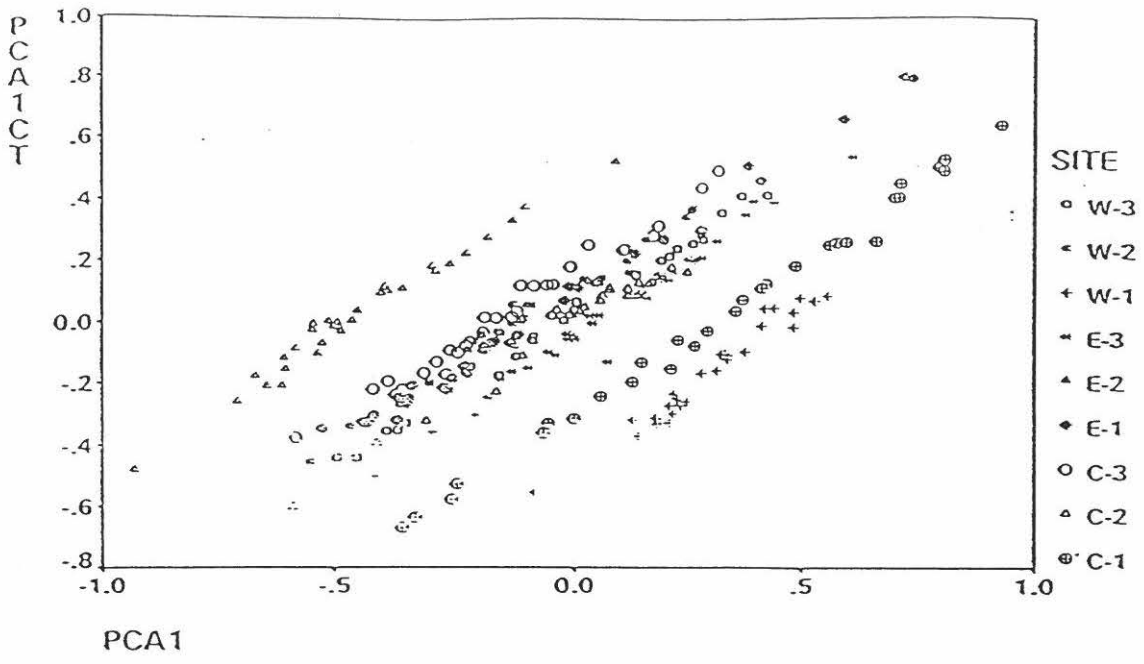

Figure 5. Relationship Between PCA-1CT (within-Population Size S) and PCA-1 (PC-1) Showing Size Differences Among the Populations of S. Mossambicus Under Study.

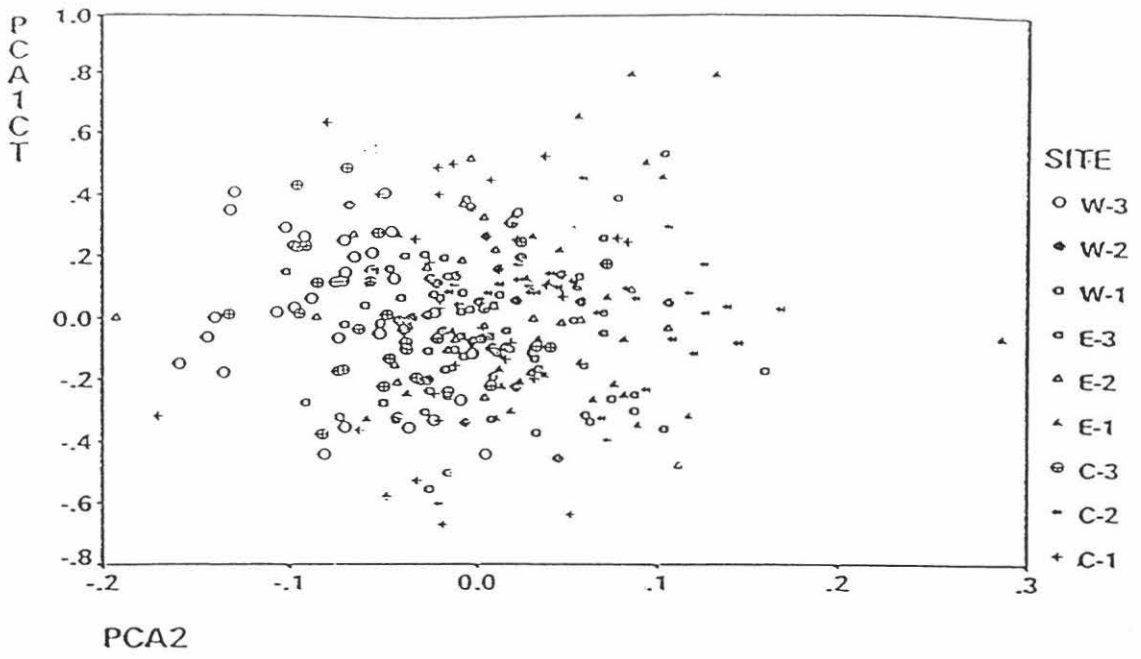

Figure 6. Relationship Between PCA-1CT (within-Population Size S) and PCA-2 (PC-2) Showing Overlapping Groups Among the Populations of S. Mossambicus Under Study.

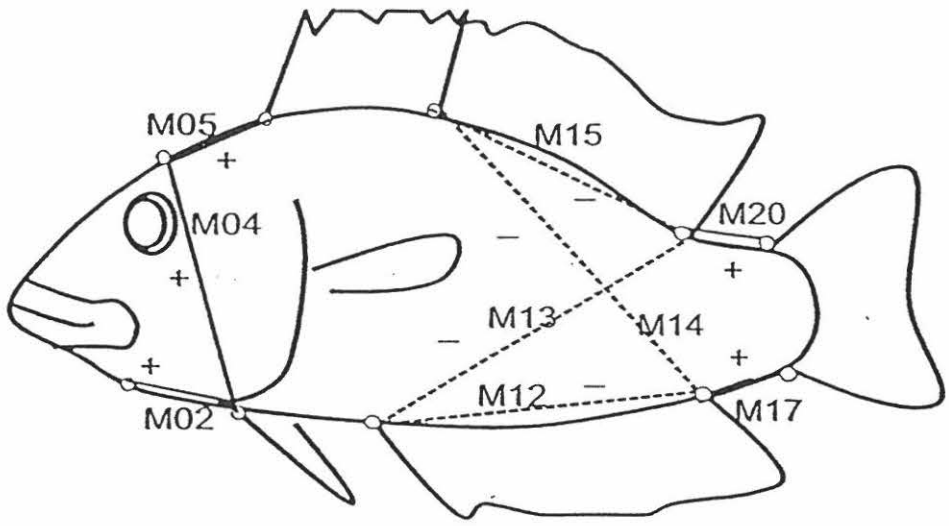

Figure 7. The Shape Indicator Variables: solid lines with positive loadings (+); dashed lines with negative loadings (-).

or individual scores on H imply a relatively long and deep head with long tail base in conjunction with a relatively shallow and short posterior abdomen. On the other hand, a high negative mean or individual score on H indicates a relatively short and shallow head with a short tail base in conjunction with a relatively long posterior abdomen.

Figure 8 shows the box plot of H for each population and reveals the shape variation within and between-populations. Analysis of variance on H (Table 6a) shows significant shape differences among the populations ($F = 20.80$; $p < 0.01$). The location of pairwise differences is indicated in Table 6b. When the recorded environmental factors are qualitatively categorized (Table 5c), correlation between the combined environmental factors and shape is significant (Table 5d: $r = 0.673$; $p = 0.047$). This result suggests that environmental factors contribute to the shape differences among the populations. Figure 9 shows the size-corrected average shape of each population reconstructed according to the method of Strauss and Bookstein (1982).

The unsheared PC-2 accounted for only 3.16% of total variation, and thus, the shape factor H accounted for even less than 3.16% of the total variation. The relationship

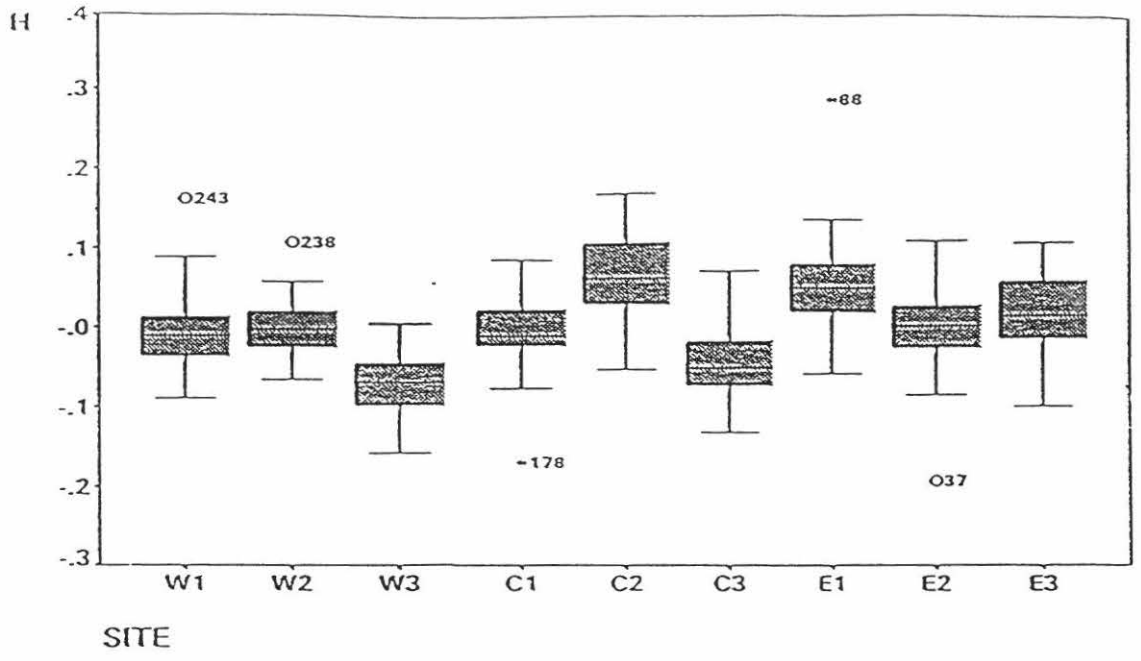

Figure 8. Box Plot of H (sheared PC-2, the Shape Variable) by Population Site. Circles and asterisks denote outliers.

Table 6a. Oneway Anova for H by Population

Source	df	SS	MS	F	P
Between Populations	8	0.4431	0.0554	20.795	0.000
Within Populations	261	0.6952	0.0027		
Total	269	1.1383			

Table 6b. Scheffé Post-test for H by Population [1)]

Pop.	Population								
	W-3	C-3	C-1	W-1	W-2	E-2	E-3	E-1	C-2
W-3									
C-3									
C-1	*								
W-1	*								
W-2	*								
E-2	*								
E-3	*	*							
E-1	*	*	*	*	*	*			
C-2	*	*	*	*	*	*			

note: 1) * means significant at 0.05 level

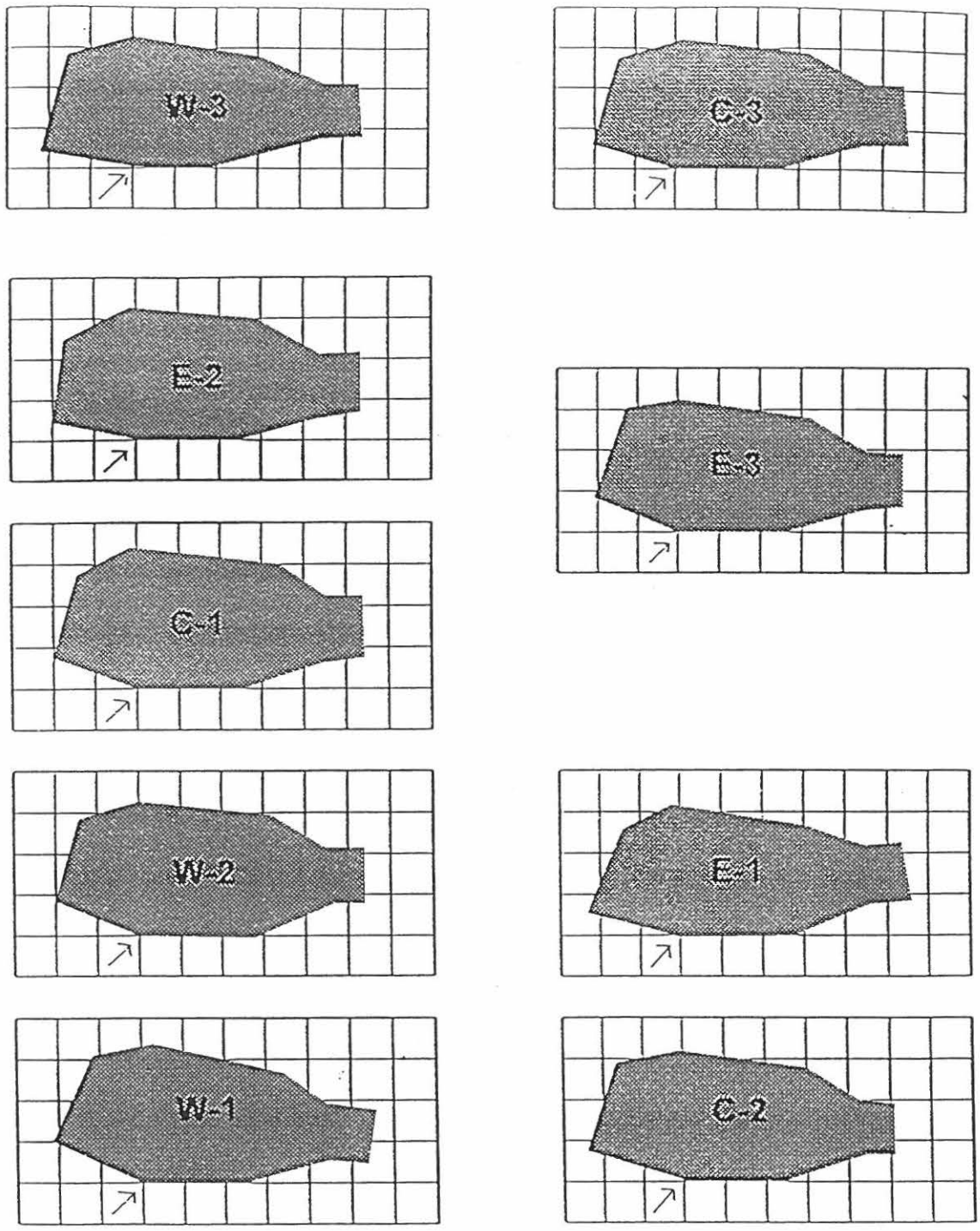

Figure 9. The Average Shapes of S. mossambicus Populations Under Study. Arrows denote anchored points.

between size and shape presented in Figure 10 also fails to show clear population separation in terms of shape.

PC-3, which accounted for 2.29% of the total variation, was also sheared to remove the within-population size component (S). After shearing, PC-3 became H' and was no longer correlated to S (Table 4). The box plot of H' for each population (Figure 11) and the analysis of variance on H' (Table 7) reveal that H' is uniform across the populations. There are no significant differences in shape among the populations represented by H'. Therefore, H' does not provide any additional information for the discrimination of populations.

Discriminant Function Analysis (DFA) on the original truss measures gave eight canonical functions for discriminating among populations. The factor loadings or the correlation between the canonical functions and the initial variables (Table 8) reveal that all of the variables have their largest absolute correlations with function 1, except variable M15 that has its largest correlation with function 4. This observation implies that most of the variables contribute in the variation among the populations, particularly because function 1 is the best discriminating function. Compared to the PCA results, function 1 can be

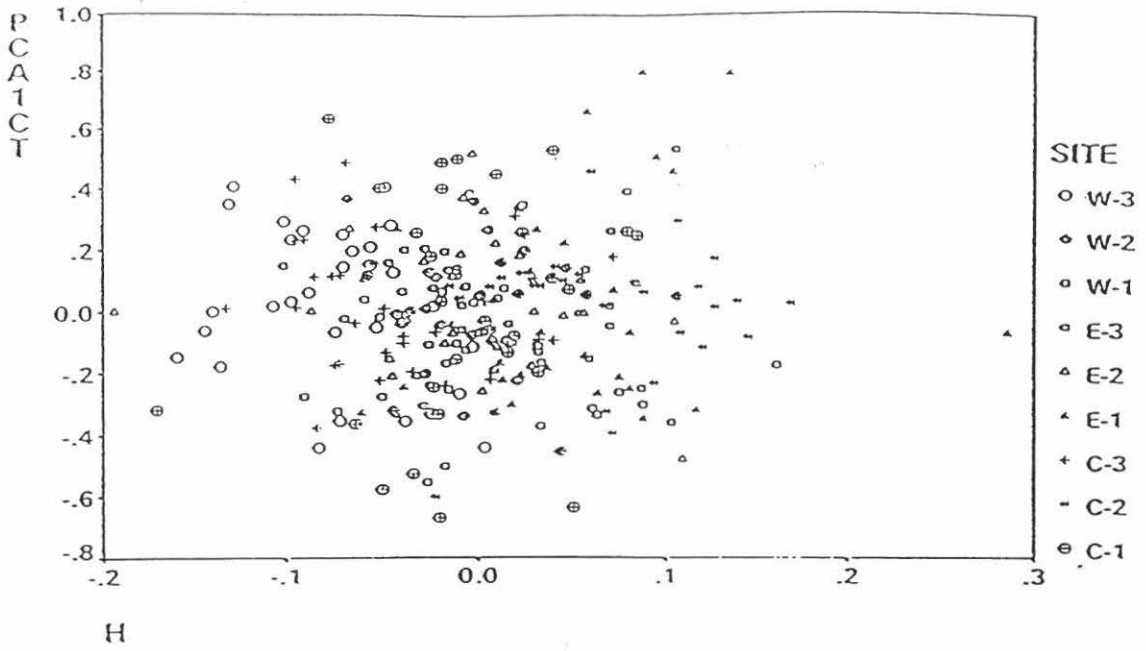

Figure 10. Relationship Between PCA-1CT (within-Population Size S) and H (sheared PC-2, the Shape Variable) Showing Overlapping Groups Among the Populations of S. Mossambicus Under Study.

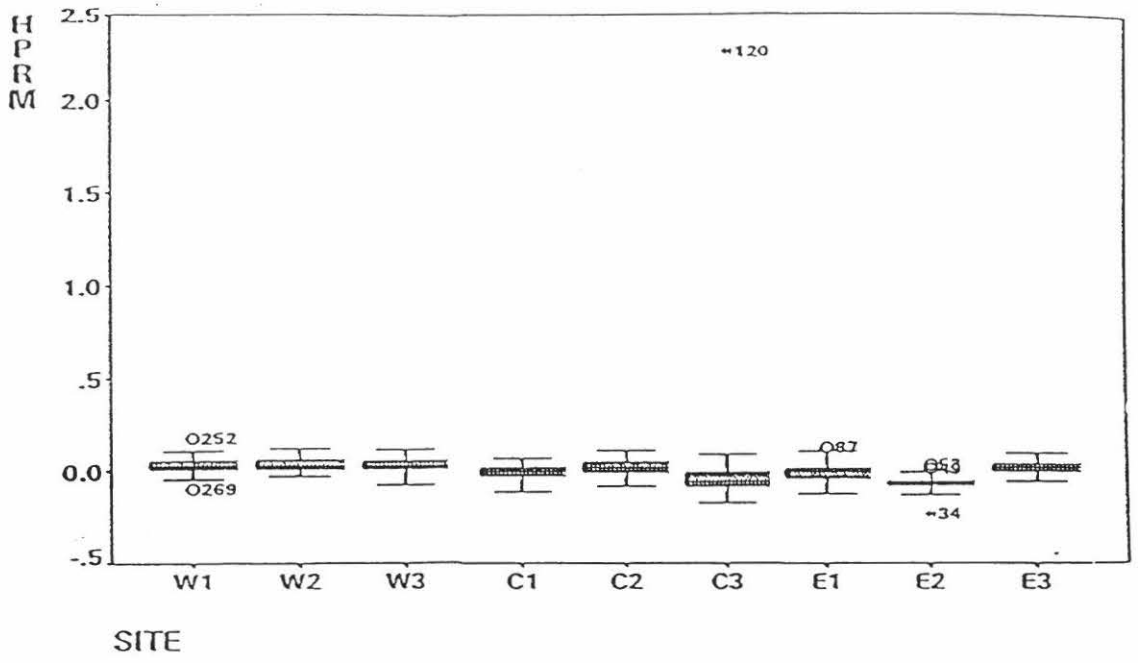

Figure 11. Box Plot of H-PRM (sheared PC-3, the Shape Variable) by Population Site. Circles and asterisk denote outliers

Table 7. Oneway Anova for H' by Population

Source	df	SS	MS	F	P[1]
Between Populations	8	0.2599	0.0325	1.453	0.175
Within Populations	261	5.8376	0.0224		
Total	269	6.0975			

note: 1) P-value greater than 0.05 means that no 2 populations are significantly different at the 0.05 level.

Table 8. Factor Loadings of the Canonical Discriminant Funtions[1)]

Var.	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8
M01	0.568*	0.081	0.219	0.370	0.164	0.136	-0.268	0.004
M02	0.555*	0.381	0.267	0.212	-0.004	-0.026	0.020	0.236
M03	0.602*	0.093	0.058	0.246	0.234	0.037	-0.158	-0.094
M04	0.740*	0.124	0.170	0.313	0.045	0.016	-0.103	0.157
M05	0.442*	0.075	-0.137	0.161	-0.030	-0.088	0.046	-0.074
M06	0.774*	-0.004	0.141	0.235	0.119	0.070	-0.071	0.075
M07	0.581*	0.013	0.214	0.350	0.154	0.270	0.294	-0.329
M08	0.738*	0.018	0.077	0.297	0.211	0.154	0.059	-0.099
M09	0.737*	0.014	0.163	0.328	0.055	-0.013	0.080	-0.086
M10	0.738*	0.100	0.074	0.312	0.180	-0.013	0.062	-0.001
M11	0.740*	-0.063	0.009	0.321	0.048	0.111	-0.036	-0.021
M12	0.475*	-0.025	0.052	0.375	0.324	0.016	0.034	0.106
M13	0.673*	-0.020	0.009	0.383	0.293	0.093	0.005	0.031
M14	0.626*	-0.146	0.021	0.445	0.086	0.004	0.015	0.022
M15	0.466	-0.272	0.032	0.494*	0.048	0.033	0.121	0.163
M16	0.752*	0.014	-0.105	0.350	0.222	-0.026	0.048	0.013
M17	0.501*	0.295	-0.118	0.271	0.091	0.044	-0.009	-0.171
M18	0.677*	0.105	-0.072	0.388	0.160	0.024	0.022	-0.102
M19	0.566*	0.124	-0.098	0.402	0.010	0.112	-0.036	0.046
M20	0.471*	0.221	-0.197	0.211	-0.040	0.320	0.055	0.235
M21	0.655*	0.015	-0.021	0.529	0.114	0.090	-0.008	-0.053

note: 1) '*' denotes largest absolute correlation between each variable and any discriminant function.

interpreted as the size discriminating function similar to PC-1.

The DFA in this study was able to correctly classify 70% of the cases, while by chance alone, this analysis would correctly classify only about 12% of the cases. Looking at the within group classification (Table 9), 90% (the highest percentage across all groups) of the cases in population E-2 were correctly classified. This DFA indicates that population E-2 is the most distinct population. According to the PCA results, population E-2 is the group with the smallest body size and is significantly different from the other size groups. Population C-3 and E-3 are similar to one another based on the DFA conclusion that 10% of individuals classified as population C-3 are from population E-3. Population C-3 and E-3 are also similar in size by PCA. The same explanation can be presented between population W-2 and W-3, that both populations are similar based on DFA and are within the same size group with PCA. Most of the misclassifications by DFA are apparently due to the size similarity already noted by PCA. This fact, which is confirmed by the χ^2-test of independence (Table 10), again shows that size differences are especially important in the DFA's discrimination among groups.

Table 9. Classification of Cases into Population Group through Discriminant Function Analysis [1)]

Actual Pop.	Pop size (n)	Predicted Population Membership								
		W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
W-1	30	19 63.3%	1 3.3%	1 3.3%	3 10.0%	4 13.3%	0 0.0%	0 0.0%	0 0.0%	2 6.7%
W-2	30	2 6.7%	21 70.0%	1 3.3%	0 0.0%	1 3.3%	0 0.0%	1 3.3%	1 3.3%	3 10.0%
W-3	30	1 3.3%	3 10.0%	21 70.0%	0 0.0%	1 3.3%	1 3.3%	1 3.3%	2 6.7%	0 0.0%
C-1	30	3 10.0%	1 3.3%	0 0.0%	24 80.0%	0 0.0%	0 0.0%	0 0.0%	1 3.3%	1 3.3%
C-2	30	1 3.3%	1 3.3%	0 0.0%	0 0.0%	23 76.7%	0 0.0%	3 10.0%	1 3.3%	1 3.3%
C-3	30	1 3.3%	0 0.0%	2 6.7%	0 0.0%	0 0.0%	20 66.7%	0 0.0%	4 13.3%	3 10.0%
E-1	30	0 0.0%	0 0.0%	0 0.0%	0 0.0%	3 3.3%	1 3.3%	20 66.7%	1 3.3%	5 16.7%
E-2	30	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	1 3.3%	2 6.7%	27 90.0%	0 0.0%
E-3	30	2 6.7%	3 10.0%	1 3.3%	0 0.0%	2 6.7%	1 3.3%	7 23.3%	0 0.0%	14 46.7%

note: 1) Percent of cases correctly classified into actual population is 70.00%.

Table 10. Test of Independence for DFA (=Observed) and PCA (=Expected) Results

Pop.		Distribution of Individuals									χ^2
		W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3	
W-1	Obs.	19	1	1	3	4	0	0	0	2	18.69*
	Exp.	10	0	0	10	0	0	0	0	10	
W-2	Obs.	2	21	1	0	1	0	1	1	3	15.49
	Exp.	0	10	5	0	5	0	5	0	5	
W-3	Obs.	1	3	21	0	1	1	1	2	0	15.49
	Exp.	0	5	10	0	5	5	5	0	0	
C-1	Obs.	3	1	0	24	0	0	0	1	1	18.80*
	Exp.	10	0	0	10	0	0	0	0	10	
C-2	Obs.	1	1	0	0	23	0	3	1	1	9.28
	Exp.	0	5	0	0	15	0	5	0	5	
C-3	Obs.	1	0	2	0	0	20	0	4	3	6.46
	Exp.	0	0	5	0	0	20	0	0	5	
E-1	Obs.	0	0	0	0	3	1	20	1	5	4.77
	Exp.	0	0	0	0	5	5	15	0	5	
E-2	Obs.	0	0	0	0	0	1	2	27	0	3.27
	Exp.	0	0	0	0	0	0	0	30	0	
E-3	Obs.	2	3	1	0	2	1	7	0	14	6.74
	Exp.	3.75	3.75	3.75	0	3.75	3.75	3.75	0	7.5	

Note: χ^2 (0.05;8) = 15.50 ; χ^2 (0.01;8) = 20.1

DISCUSSION

Variation in size across populations does not show a clinal or other clear geographic pattern (Figure 4). It suggested that local rather than broad-scale regional factors are controlling size.

One possible explanation for the cause of small body size in population E-2 is that this population is near Sidoarjo and may have a poorer environment as compared to the other populations. Poor water quality in this locality may result from the densely populated urban city of Sidoarjo that is also very close to the industrial city of Surabaya (the second most populated area in Java). Shallow water subjected to more frequent water level fluctuation may also contribute to creating unfavorable conditions for the fish in this site. Moreover, in a small water body, prolific reproduction of the fish may greatly increase the population density resulting in competition and small body size due to lack of food supply (Hepher and Pruginin, 1982). Neoteny (the ability of fish to breed successfully while still at juvenile state due to adverse environmental conditions) may also give different truss values because the shape of the fish varies with size in allometric growth.

Different physicochemical factors in the other sampling sites include the cooler water temperature experienced by population E-1 from Sarangan and population W-2 from Situ Bagendit; the warmer but more extreme in water level fluctuations, such as in population C-3 from Rembang and population W-3 from Losari; warmer water with less water fluctuations but smaller water body such as in population C-2 from Magelang; warmer, less fluctuating water fluctuation and larger water body such as in population E-3 from Situbondo; deeper and more stable water body, and warmer temperature such as in population C-1 from Pekalongan and population W-1 from Serang. Correlations between the recorded environmental factors and size are not significant (Table 5d). However, some other factors which are not recorded such as food availability and the chemistry of the waters that cause local differences may have influenced the size variation of the fish.

According to Le Roith (1991), the growth and development of organisms are regulated by cell to cell communication performed by hormones and other growth factors. The hormones such as growth hormones, insulin and estrogens are secreted by classical endocrine glands, while growth factors are produced by numerous tissues such as the kidney and liver. Many different growth factors have been

discovered in vertebrates such as the insulin-like growth factors (IGFs) that mediate the growth promoting effects of growth hormones. The growth itself is not indefinite, since the genetic background of an individual sets the potential limits for maximum growth. Further, an individual placed in adverse environmental conditions may not be able to achieve its full size potentially achieveable by its genetic makeup (Ferro-Luzzi, 1984; Schell, 1984). The reduced growth represented by smaller body size is regarded as a response of the individual to adverse environmental conditions. This plasticity can also be viewed as a strategy of the organism for adaptation in order to minimize environmental stress. Ajit Ray (1984) stated that a particular gene may have different phenotypic effects in different environmental conditions. Similarly, Dunham et al. (1990) conducted a study on genetic-environment interaction for growth of catfish stocks and found that a genetic stock that grows better in one set of environmental conditions might not be the best in another set of environmental conditions.

Applied to S. mossambicus, Lowe-McConnel (1982) stated that the largest individual ever reported was from Lake Rudolf in Africa with a total length of 64 cm. Her studies on tilapia growth suggested that environmental factors

greatly affect the maximum size of the fish. The same species of tilapia will grow at different rates in different bodies of water. This statement suggests that environmental differences can be more potent than genetic differences in determining maturation and maximum size. Maturation time and maximum size tend to be smaller in small bodies of water than in larger ones. Lowe-McConnel (1982) further stated that tilapias apparently have biological properties that allow them to switch their energy budget from growth to reproduction in unfavorable conditions, and thus maturation is achieved at a younger age. Feeding activities normally stop after reproduction that occurs almost every month in tropical waters. Therefore, the maximum size of this fish in tropical waters is also achieved at the time when the fish start spawning.

Noakes and Balon (1982) stated that environmental stress factors include food supply, water temperature, chemical substances or fluctuating environmental conditions. Among these stress factors, physicochemical types of signals are stronger than stimuli related to food in affecting the biology of tilapias. In the presence of abundant food, at an optimum water temperature of 25° C, tilapias may begin to reproduce at about 4 cm standard length in aquaria. Stable conditions over relatively long time periods in a large body

of waters such as in a lake, will lead to a longer growth intervals, delayed maturation and decreased fecundity with larger eggs, producing larger maximum size. The super-ability for tilapias to adapt to local conditions, even in shallow water bodies subject to water level fluctuations, is in part responsible for their widespread distribution and success as colonizers.

A great plasticity of growth and reproductive characteristics are commonly observed among tilapia populations in natural waters. Noakes and Balon (1982) and Pullin (1982) noted that plasticity is a phenotypic phenomenon which is reversible. An example of this characteristic was a case in which S. mossambicus bred 2 to 3 times over a 2-year period in small aquaria achieving a maximum size of only about 50 g. When transferred to a more spacious environment in the farm, the fish stopped breeding until they weighed about 250 g at 3 years of age. Romana-Eguia and Doyle (1992) and Weatherly and Gill (1987) also confirmed that growth retardation caused by substandard rearing conditions such as starvation, low rations, suboptimal diet, etc., does not significantly hamper subsequent growth when the fish are restored to normal culture conditions.

In this study, body size variation among the tilapia populations could be due to ecophenotypic and/or genetic factors. However, the results obtained from isozyme and RAPD data that will be discussed later in this tilapia study do not support a strong genetic determinism for the observed size differences. In this study, populations which are different by morphometric data are not those that are different by protein and DNA analysis. These results are similar to those obtained by Yoshiyama and Sassaman (1983) when comparing morphological and allozymic samples of stichaeid fish collected from open coast localities from Alaska to California. Lack of a significant relationship between enzyme and morphological traits in this stichaeid study was due to appreciable environmental (non genetic) influence on the morphological characters examined.

The above discussion may inductively lead to a conclusion that the size variation among S. mossambicus populations in this study is due to plasticity in adaptation to local environment. This tentative conclusion could be confirmed by doing further study on the reversibility of size when transfers are made among the populations. If body size is not 'reversible' by the transfers, it can then be concluded that size differentiation is controlled by non environmentally induced factors. In this study, individuals

from population C-1 and W-1 were comparatively large. Further study should also be done to determine the set of environmental conditions of population C-1 and W-1 in which S. mossambicus grows to a larger size. The information on the salient differences in environmental conditions might provide insight into simulating or modifying conditions for the most efficient tilapia farming.

Pante (1988) recommended the analyses of shape rather than size to discriminate populations. Shape is preferred to size because it has ramifications for characters used in systematic studies (Wimberger, 1992). Shape differences among populations of the Javanese tilapia in this study, although statistically significant, are very small. The types of body shapes found among these populations are somewhat similar to those of Cichlasoma citrinellum studied by Meyer (1990). Meyer (1990) found two types of body shapes: the elongate limnetic body form in individuals that feeds mostly on soft materials, and the stouter benthic body form in individuals that feeds on harder materials such as snails. Tilapia of population W-3 from Losari-West Java are comparable to the limnetic body form of C. citrinellum. The fish of population E-1 (Sarangan-East Java) and population C-2 (Magelang-Central Java) are similar to the benthic body form of C. citrinellum. Tilapia population C-3 from

Rembang-Central Java looks somewhat like population W-3, the limnetic body form, while the population E-3 from Situbondo-East Java looks more like the benthic body form. Fish population E-2 of Sidoarjo-East Java, population C-1 of Pekalongan-Central Java, population W-2 of Situ Bagendit-West Java and population W-1 of Serang-West Java are intermediate between the two extremes.

Similar to size, plasticity in shape is also common among fish populations (Bookstein et al., 1985). The divergent patterns of growth among conspecific populations existing in different habitats may lead to different body shapes. An example of ecophenotypic or environmentally-induced differentiation is the clear morphological differences between freshwater sculpin (Cottus cognatus) from Lakes Cayuga and Ontario in New York and those from streams in Michigan and Pennsylvania (Strauss, 1980). In a study on the zoogeography of spotted sunfish (Lepomis punctatus) in North America, Warren (1992) also found 2 distinct morphological groups within this species complex. He then presented 2 taxonomic ranking alternatives as follows : a) Using the biological species concept (BSC), the 2 epiphenotypes are assigned as 2 subspecies (L. p. punctatus and L. p. miniatus) under a single polytypic biological species, due to the presence of a hybrid zone

between the 2 groups; and b) Using the evolutionary species concept (ESC), the 2 epiphenotypes can be ranked as 2 separate species (L. punctatus and L. miniatus).

According to West-Eberhard (1986), morphological plasticity in fishes facilitates a rapid rate of speciation as species are buffered against extinction. Meyer (1990), however, had a contrasting view that plasticity may buffer the action of selection and thus inhibits morphological evolution. He further stated that morphological plasticity in combination with a fluctuating environment may cause remarkable morphological variation. The adaptive differences in external morphology may eventually lead to genetic isolation.

The small differences in shapes among Javanese tilapia populations in this study can most likely be attributed to morphological plasticity of the fish. This conclusion is also consistent with the following findings : a) correlation between recorded combined environmental factors and shape groups is significant (Table 6c and Table 6d: $r = 0.673$ with $p = 0.047$); b) polymorphism in shape is known to occur in cichlid species (Greenwood, 1965; Strauss, 1980; Bookstein et al., 1985; Witte, 1984; Meyer, 1990); c) the external morphology of fishes in general are thought to be phenotypically plastic and are susceptible to environmental

influences such as diet, development, growth rate and nutrition (Allendorf et al, 1987; Meyer, 1987; Meyer, 1990); d) shape shifts due to morphological plasticity can occur even within environmental changes lasting only 3 to 8 months (Meyer, 1990; Wimberger, 1992); e) the dispersal period of tilapia in Java, which has been only about 55 years (Philippart and Ruwet, 1982), may be too short to bring about morphological evolutionary divergence among the populations; f) there was no distinct epiphenotype based on shape could be determined among S. mossambicus populations in this study.

CHAPTER THREE

ISOZYME STUDY OF S. mossambicus POPULATIONS IN JAVA

INTRODUCTION

Starch gel electrophoresis was first demonstrated by Oliver Smithies in 1955 through his work on the separation of proteins from human tissue extract (Smithies, 1955). Enzyme activities on the gel were determined after extracting and eluting the enzymes from the gel. Shortly after this study, Hunter and Market (1957) introduced histochemical methods to uncover specific enzymes on gel slices in situ. Since then, starch gel electrophoresis coupled with histochemical staining has become a rapid method for separating and visualizing proteins, and is widely used to reveal genetic variations in ontogenetic, phylogenetic and biogeographic studies of plant and animal populations (Buth, 1990).

Protein electrophoresis used in ontogenetic studies can detect the spatial and temporal patterns of gene expression during development. Since those expression schedules vary across the taxa, detection of altered expression schedules (heterochromic shifts) in hybrid individuals is important

for assessing the regulatory divergence of the parental taxa. Protein electrophoresis can also identify the effect of genetic polymorphism on the fitness traits such as rates and stability of development. Thus, this kind of ontogenetic study can be used for the management of species.

Protein electrophoresis has also become a dominant method for estimating genetic variability within and among populations of organisms. Such studies have provided important information about population structure and stock identification with management and conservation implications.

Protein electrophoresis studies have been conducted to compare the population genetics among various populations of fishes. Genetic differentiation among wild populations of fishes has been reported on populations of brown trout (Crozier and Ferguson, 1986; Allendorf et al., 1977), Atlantic salmon (Sanchez et al., 1991; Stahl, 1987), fourhorn sculpin (Gyllensten and Ryman, 1988), and even penaeid shrimp (Sunden and Davis, 1991) and oyster (Gaffney et al., 1992; Hedgecock and Sly, 1990).

Shaklee et al., (1990) identified and defined the boundaries of the stocks of Spanish mackerel, Australian baramundi and Pacific salmon using protein electrophoresis. Considerable effort has been expended to find protein

markers for stock identification of tilapia, including hybridization and introgression studies of these species (Chen and Tsuyuki, 1970; Cruz et al., 1982; Brummet et al., 1988a; De Silva and Ranasinghe, 1989). Stock comparisons and hybrid identifications among carp populations have also been done by protein electrophoresis (Sumantadinata and Taniguchi, 1990; Brummet et al., 1988b; Magee and Philipp, 1982; Campton, 1990).

Zoogeographical or clinal genetic variations can be detected using protein electrophoresis. Such studies have been done in killifish (Powers, 1990), cockscomb (Sassaman et al., 1983), stichaeid fish (Sassaman and Yoshiyama, 1979; Yoshiyama and Sassaman, 1983), and sculpin (Yoshiyama and Sassaman, 1987).

Protein data can also be used for phylogenetic and related taxonomic studies. The following are examples of such broad applications of protein electrophoresis: an inheritance study of red body coloration in tilapias (Wohlfarth et al., 1990); the heredity of sex determination in tilapias (Wohlfarth and Wedekind, 1991); speciation processes (Renaud et al., 1986; Verheyen et al., 1985; Ovenden and White, 1990); genetic relationship among tilapia species (Oosthuizen et al., 1993); genetic differentiation due to domestication in brown trout (Garcia-Marin et al,

1991); and genetic divergence among congeneric species (Grant, 1987).

Utter et al. (1987) mentioned some strengths and limitations of electrophoretic data for studying protein loci. The procedure is relatively cost effective. Protein extracts can be easily prepared; the materials needed are relatively cheap, and a large volume of data can be collected in a relatively short period of time. However, the electrophoretic results that show protein identity do not necessarily mean identity in DNA sequences. Therefore, protein data are less sensitive in detecting genetic variation than are nucleic data. Another important limitation of protein electrophoresis is the appearance of shadow bands as an effect of the length of time and conditions under which the samples were stored; this may become a serious problem for some loci. In order to avoid these artifacts, collection on dry ice, storage at low temperature (-80°C) and analysis within a few weeks of collection are recommended.

Despite the limitations mentioned above, protein electrophoresis is still chosen for most studies of variation in natural populations, especially because of economical reasons (May and Krueger, 1990). Morizot and Schmidt (1990) also stated that protein-level technologies

should not be replaced by nucleic acid studies, because the future laboratories should have proficiencies in analyzing both genes and their products. Starch gel electrophoresis will continue to be used for initial screening of genetic variability.

The main objective of this study is to measure genetic variability among S. mossambicus populations from Java and to identify any population substructuring. Such information is useful for management and conservation purposes.

MATERIALS AND METHODS

Sample Collection

Tissue samples of S. mossambicus collected for morphometric study were also used for enzyme electrophoresis. Tissue samples from liver, eye and muscle were separately put in 1.5 ml microfuge tubes to which 0.5 ml of 0.25M Tris pH 7.0 as the homogenizing buffer was added (Abdelhamid, 1988). All tissue samples were kept on dry ice before and during transportation to the laboratory where they were immediately stored at -86°C (Baverstock and Moritz, 1990).

Data Collection

Starch gel electrophoresis and the staining procedure followed the protocols from Abdelhamid (1988), Wendel and Weeden (1989), Shaw and Prasad (1970) and Morizot and Schmidt (1990). The entire data collection consisted of 2 stages, the preliminary or pilot study and the primary study.

The preliminary study was aimed to find resolvable and/or polymorphic enzyme loci suitable for this study, and at the same time to perform optimization of electrophoretic conditions in terms of buffer system, pH, gel concentration and tissue selection.

Several concentrations of gel ranging from 9.0 to 12.5% (Murphy et al., 1990; Morizot and Schmidt, 1990; McAndrew and Majumdar, 1983) were first tried. From these experiments, a gel concentration of 11.5% was considered best in terms of resolution quality, ease of handling and running time.

In the preliminary study, 26 enzyme systems were surveyed using 2 buffer systems at 5 different pH's and 3 different tissues (Table 11). These experiments resulted in the selection of a total of 16 enzyme systems that gave 27 loci using 2 buffer systems at different pH and from different tissues, as presented in Table 12.

Table 11. Enzyme and Buffer Systems Tested During the Preliminary Study*)

Locus	Tris-EDTA-Borate (TBE)				Tris-Citrate (TC)				
	pH8.6/A	pH8.5/MA	pH8.0/MS	pH8.0/S	pH6.8/A	pH8.0/MA	pH7.0/MS	pH8.0/MS	pH7.0/S
Ldh	E	E							
Mdh		E,M			E,M				E,M
Idh					M	M			M
Adh			LEM				LEM		LEM
Gdh	LEM	LEM		LEM			LEM		LEM
Gpd	LEM	LEM	LEM	LEM				LEM	LEM
Gapd	LEM	LEM	LEM		LEM				LEM
G6pd	LEM				LEM		LEM	LEM	
Pgd			LEM		LEM	LEM	LEM		
Gpi					LEM	LEM			
Mpi	LEM	LEM	LEM		LEM		LEM	LEM	
Ak						LEM		LEM	LEM
Ck						LEM	LEM	LEM	LEM
Me	M					M			
Sod	LEM	LEM							
Fum	LEM	LEM	LEM	LEM	LEM		LEM	LEM	
Pgm						LEM		LEM	LEM
Fbp	L,M	L,M	L,M		L,M		L,M		
Aco	LEM	LEM	LEM		LEM		LEM	LEM	
Acp					LEM				
Ap			LEM		LEM		LEM		
Catalas			LEM		LEM				
Aat			LEM		LEM		LEM	LEM	
Est			LEM				LEM		
Est-D			LEM				LEM		
ES	LEM	LEM				LEM			

Table 11 (Continued):

*) note:

A: Abdelhamid (1988)
MA: McAndrew and Majumdar (1983)
MS: Morizot and Schmidt (1990)
SP: Shaw and Prasad (1970)

L: Liver
E: Eye
M: Muscle
LEM: Liver, Eye and Muscle

Table 12. The Systems and Conditions of Electrophoresis During the Preliminary Study [1)]

Enzyme		Buffer	pH/tissue	Run time (hours)	Staining Recipe
Glucose Phosphate Isomerase	(GPI: E.C.5.3.1.9)	TC/A	6.8/M	7	MS
Aconitase	(ACO: E.C.4.2.1.3)	TC/MS	7.0/L	4	MS
Malate dehydrogenase*)	(MDH: E.C.1.1.1.37)	TC/A	6.8/M	7	SP
Lactate Dehydrogenase	(LDH: E.C.1.1.1.27)	TBE/A	8.6/E	8	SP
Isocitrate Dehydrogenase	(IDH: E.C.1.1.1.42)	TC/A	6.8/M	7	SP
Malic Enzyme	(ME: E.C.1.1.1.40)	TBE/A	8.6/M	10	MS
Fumarase	(FUM: E.C.4.2.1.2)	TBE/A	8.6/M	10	MS
Alcohol Dehydrogenase	(ADH: E.C.1.1.1.1)	TC/MS	7.0/L	4	SP
Phospho-Gluconate Dehydrogenase	(PGD: E.C.1.1.1.43)	TBE/A	8.6/M	10	MS
Mannose Phosphate Isomerase	(MPI: E.C.5.3.1.8)	TBE/A	8.6/M	10	MS
Phospho-Gluco-Mutase	(PGM: E.C.2.7.5.1)	TC/MS	8.0/M	5	MS
Super-Oxide Dismutase	(SOD: E.C.1.15.1.1)	TBE/A	8.6/M	10	MS
Adenylate Kinase	(AK: E.C.2.7.4.3)	TC/MS	8.0/E	5	MS
Creatine Kinase	(CK: E.C.2.7.3.2)	TC/MS	8.0/E	5	MS
Esterase	(EST: E.C.3.1.1.1)	TBE/A	8.6/L	8	SP
Esterases, others	(ES: E.C.3.1.1.1)	TBE/MS	8.0/L	8	MS

1) note: L = liver; E = eye; M = muscle
TC = Tris-Citrate Buffer
TBE = Tris-Borate-EDTA Buffer
A = Abdelhamid (1988)
MS = Morizot and Schmidt (1990)
SP = Shaw and Prasad (1970)

*) : The 4 loci of Mdh in this study :
Mdh-E1, Mdh-E2 and Mdh-E3 were from eye;
Mdh-M4 was from muscle tissue.

In the primary study, 25 individuals from each population (Nei, 1983) were used for each enzyme system in every electrophoretic run.

The general procedure for each electrophoretic run consisted of the following steps:

Gel Preparation. About 1/4 volume of gel buffer was first added to the already weighed starch in an Erlenmeyer flask. This concentrated solution was continuously swirled so that the starch did not precipitate. The remaining 3/4 volume of the gel buffer was put in another flask and heated in a microwave until almost boiling, then added into the starch solution. The colloidal starch solution was thoroughly mixed and returned to the microwave. This solution was heated in the microwave and was taken out of the microwave every 15 seconds and swirled. When exactly boiling, the solution was degassed using a vacum pump, then poured into a prepared electrophoretic tray. When the gel reached room temperature, it was covered with plastic wrap and refrigerated at 4°C for about an hour for gel stabilization (Morizot and Schmidt, 1990).

Sample Homogenization. The whole tissue sample in its microfuge tube was taken from the -86°C freezer and thawed. Tissue samples were then ground individually to break the cells. An electric drill equipped with a special plastic

pestle to fit the microfuge tube was used to grind cells. A sample of the resulting homogenate, which was expected to contain the enzymes, was absorbed onto a 4mm x 12mm piece of filter paper, which served as the enzyme source for the electrophoretic run.

Sample Loading and Running the Electrophoresis. Wells on the gel were made by making a slit or cut from one side to the other of the gel using a thin spatula. The filter paper piece containing enzyme samples was then inserted within the slit using a pinset. Every gel could accomodate 25 samples plus 3 standards from known pure tilapia species and another filter paper containing methylene blue solution for a dye marker. Electrophoresis was performed in a 4°C refrigerated chamber. The run was completed when the blue marker reached a distance of 12 cm from the wells. The average running time varied depending on the buffer system (Table 12).

Gel Slicing and Staining. When the run finished, the gel was sliced on a slicing board using a streched fine metal string (a guitar E string) so that each slice had a thickness of approximately 1.5 mm. Each slice was then stained for isozyme content in a staining tray. The recipes of the histochemical staining mixtures were taken from different sources as shown in Table 12.

Gel Fixing and Preservation. Gel fixation was done to stop the staining reactions so that the isozyme bands were not overdeveloped. The fixing solution was Smithies's solution which also functioned as a preservative. The solution was a mixture of water, methanol, glycerol and glacial acetic acid at the proportion of 5 : 5 : 2 : 2. The gel was first washed several times with deionized water until it was cleaned of the remaining staining mixture, then covered with the fixing solution. Finally, the gel was taken out of the fixing tray, plastic wrapped and kept in the refrigerator until scoring.

Scoring and Photodocumentation. The interpreting and scoring of the isozyme banding pattern of each locus was done on an illuminated board. By scoring each band, the number of alleles present in each population could be determined for each locus. Then, the genotype as well as gene frequencies for each locus that occured within each population were calculated and recorded. Documentation was completed by photographing each gel with 35mm film. If more than 1 locus was observed for an enzyme system, the most cathodally migrating locus was assigned as locus 1 and the slowest allele within each locus was assigned as allele A.

Data Analysis

The overall gene frequency data were used to calculate Nei's (1983) total gene diversity H_t (expected total heterozygosity of the populations), gene diversity within population H_s (average expected heterozygosity of each population), gene diversity among populations D_{st}, and the coefficient of gene differentiation G_{st} (= D_{st} / H_t or = 1 - (H_s / H_t)). The GENESTAT-PC program version 3.3 of Lewis (1993) was employed to compute these statistics.

The coefficient of gene differentiation (G_{st}) is a measure of the degree of population divergence in the context of multiallelic loci. In diallelic systems, G_{st} is the fixation index F_{st} (Hartl and Clark, 1989). These indices have a theoretical range from 0 to 1, with higher values indicating more divergence among the populations in the study (Sunder and Davis, 1991). The F_{st} value approaches 1 if populations are fixed for different alleles. The F_{st} (= 1 - (H_s / H_t)) in this study was computed based on polymorphic loci only. Statistical significance of the population differentiation was tested with the chi-square variate of $\chi^2 = (2n)F_{st}$ or $\chi^2 = (2n)G_{st}$ with (k-1) degree of freedom, where n is the total number of individuals and k is the number of populations (Yoshiyama and Sassaman, 1983).

The standardized genetic variance F_{st} was also used to measure the heterogeneity of gene frequencies among the populations (Workman and Niswander, 1970). However, in order to know the pattern of population differentiation for each locus, gene frequency heterogeneities among populations were tested for significance using the chi-square test for independence (chi-square test of homogeneity or chi-square contingency test). The k x m contigency tables have χ^2 values with a (k-1)(m-1) degree of freedom, where k is the number of populations included in the test and m is the number of alleles of the locus in comparison (Whitkus et al., 1987; Sokal and Rohlf, 1969; Wonnacott and Wonnacott, 1985). These heterogeneity tests were performed with the microcomputer version 5.0 of SYSTAT for windows.

Nei's (1972) standard genetic distance (D) was calculated as a measure of overall differences in each pairwise population comparison, using the GENESTAT-PC 3.3 program.

The pattern of divergence among populations was then depicted in an UPGMA dendogram based on the genetic distances, and in a 3-dimensional diagram derived from Principle Coordinate Analysis (PCO analysis). The UPGMA and PCO analyses were calculated using the NTSYS-PC program version 1.40 of Rohlf (1988).

The fit of the genotype distribution to the Hardy-Weinberg equilibrium was tested using the simplified exact test (probability test) for the 2-allele case by Vithayasai (1973). This test is based on Levene's (1949) formulae for small samples. In the case of small sample size, the use of the ordinary chi-square distribution test is not appropriate because the expected number of some genotypes is very small. In addition, the inbreeding coefficient or Wright F_{is} was calculated for each variable locus within each population as $F_{is} = 1 - (H_o / H_s)$, where H_o is the observed heterozygosity within the population and H_s is the expected heterozygosity in that population. The value of F_{is} overall loci for each population or F_{is} overall populations for each locus was computed as $F_{is}' = 1 - ((\Sigma\ i=1,k\ H_o n_i)/(\Sigma\ i=1,k\ H_s n_i))$, where for overall loci calculation, n is the number of individuals at locus i and k is the number of variable loci in that population, and for the overall populations calculation, n is the number of individuals in population i and k is the number of populations at that locus (Crawford et al., 1988; Yoshiyama and Sassaman, 1983). The value of F_{is} can be interpreted as a measure of deviation from value expected under Hardy-Weinberg equilibrium within a population. The significance of F_{is} was tested using the formula of $\chi^2 = n(F_{is})^2$ with 1 degree of freedom, where n is

the number of individuals per population (Li and Horvitz, 1953). The F_{is} is positive if there is a deficit of heterozygous individuals.

Other indices such as proportion of polymorphic loci (P), number of alleles per locus (A), number of alleles per polymorphic locus (A_p), and the observed heterozygosity (H_o) for each population were calculated using GAP program of Pack (1988).

RESULTS

The 16 enzyme systems assayed produced 27 presumptive loci, of which 12 loci were polymorphic at 95%. Except for the PGD that showed 3 allelic locus, all other polymorphic loci were in 2 allelic systems. Appendix 3 and Appendix 4 present the genotype and gene frequencies, respectively, of the polymorphic loci for each population.

The degree of genetic divergence among the populations measured by the G_{st} (= F_{st}) was presented in Table 13. The average G_{st} value adjusted for sample size was 0.067, while a G_{st} = 0.085 was for the unmodified calculation. Both values are significant ($p < 0.01$), indicating the

Table 13. Gene Diversity Statistics Based on Isozymes for *S. mossambicus* Populations Under Study [1)]

Locus	Unbiased for Sample Size				Unmodified				
	H_s	D_{st}	H_t	G_{st}	H_s	D_{st}	H_t	G_{st}	$\chi^{2*)}$
Gpi-1	0.0306	0.0001	0.0307	0.004	0.0300	0.0007	0.0307	0.0219	9.86
Gpi-2	0.0306	0.0001	0.0307	0.004	0.0300	0.0007	0.0307	0.0219	9.86
Es-3	0.1170	0.0000	0.1170	0.000	0.1147	0.0023	0.1167	0.0174	7.83
Es-4	0.3666	0.0726	0.4392	0.162**	0.3593	0.1317	0.4384	0.1804	81.18**
Aco	0.0378	0.0015	0.0393	0.037*	0.0371	0.0022	0.0392	0.0544	24.48**
Ldh-3	0.0305	0.0045	0.0350	0.129**	0.0299	0.0052	0.0349	0.1448	65.16**
Idh	0.1045	0.0046	0.1091	0.042*	0.1024	0.0072	0.1089	0.0595	26.78**
Me-1	0.1951	0.0063	0.2014	0.031	0.1912	0.0122	0.2010	0.0487	21.92**
Me-2	0.2097	0.0020	0.2117	0.009	0.2055	0.0072	0.2112	0.0269	12.11
Fum	0.0256	0.0008	0.0264	0.030	0.0251	0.0013	0.0263	0.0473	21.29**
Adh	0.0239	0.0024	0.0264	0.092**	0.0235	0.0029	0.0263	0.1081	48.65**
Pgd	0.3878	0.0172	0.4051	0.043*	0.3801	0.0397	0.4042	0.0597	26.87**
Means [2)]	0.0578	0.0042	0.0619	0.067**	0.0566	0.0052	0.0618	0.0838	356.0**

note: 1) only polymorphic loci (= 12 loci) are tabulated;
H_s = gene diversity in each population;
H_t = gene diversity in the whole population;
D_{st}= gene diversity among populations;
G_{st}= F_{st} = coefficient of gene differentiation;

2) Means are calculated for overall 27 loci; the mean of χ^2 is the total χ^2 of the 12 loci with total df = 104.

*) the $\chi^2 = 2N \times G_{st} = (2)(225)(G_{st}) = 450 \times G_{st}$; df = 8; N = 9 x n; n = sample size = 25.

**) $\chi^2_{8;0.05} = 15.5$; $\chi^2_{8;0.01} = 20.1$

existence of substructuring within the S. mossambicus populations in Java.

The patterns of population differentiation for each polymorphic locus resulting from the chi-square contigency tests are presented in Table 14. The significant results of the tests are comparable to those of the unmodified F_{st}. Out of the 12 polymorphic loci, 8 loci indicated significant divergence among the populations.

Overall differences between each pair of populations measured by Nei's genetic distance (D) are presented in Table 15. The D values ranged from 0.000 to 0.023 with an average of 0.005. The overall pattern of population structure among the populations of S. mossambicus under study is given by the UPGMA phenogram in Figure 12 and PCO diagram in Figure 13. Populations E-1 and C-3 are genetically identical and both were closely related to population E-3. Population E-2 and W-2 are clustered together and both are related to population C-2, C-3, E-1 and W-1. Population C-1, C-2 and W-3 are individually separated, indicating the existence of unique genetic entities among them.

The results of the Exact Tests for the deviations from Hardy-Weinberg equilibrium are shown in Table 16. There

Table 14. Chi-square Test for Independence for the Isozyme Gene Frequency Heterogeneities Among the Populations of S. mossambicus Under Study [1)]

Locus	χ^2	df	Pattern of Population Differentiation
Gpi-1	9.87	8	non significant
Gpi-2	9.87	8	non significant
Es-3	7.85	8	non significant
Es-4	81.17**	8	E3 W3 C2 C1 E1 C3 W1 E2 W2
Me-1	21.89**	8	E3 C3 W2 E1 C1 E2 W1 C2 W3
Me-2	12.12	8	non significant
Aco	24.49**	8	E3 E2 E1 C3 C1 W1 W3 W2 C2
Ldh-3	65.16**	8	W3 E3 E2 E1 C3 C2 C1 W2 W1
Idh	26.78**	8	E2 C1 W3 W2 C3 E3 E1 C2 W1
Fum	21.28**	8	E3 E2 E1 C3 C2 W2 C1 W1 W3
Adh	48.65**	8	C1 E3 E2 E1 C3 C2 W3 W2 W1
Pgd	54.89**	16	C1 W1 E3 E2 E1 C3 C2 W3 W2
Total	384.02**	104	See : Figure 12 (UPGMA dendogram) and Figure 13 (PCO diagram)

note: 1) population names are referred to geographical sites as shown in the map (Figure 1); population clusters are denoted by lines.

Table 15. Nei's Standard Genetic Distances Based on Isozymes Among S. mossambicus Populations Under Study

Pop	Populations								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
W-1	–	–	–	–	–	–	–	–	–
W-2	0.0128	–	–	–	–	–	–	–	–
W-3	0.0034	0.0227	–	–	–	–	–	–	–
C-1	0.0008	0.0136	0.0039	–	–	–	–	–	–
C-2	0.0004	0.0207	0.0021	0.0020	–	–	–	–	–
C-3	0.0014	0.0114	0.0045	0.0004	0.0020	–	–	–	–
E-1	0.0003	0.0103	0.0039	0.0006	0.0015	0.0000	–	–	–
E-2	0.0015	0.0110	0.0034	0.0016	0.0025	0.0002	0.0006	–	–
E-3	0.0029	0.0226	0.0029	0.0023	0.0017	0.0018	0.0015	0.0035	–

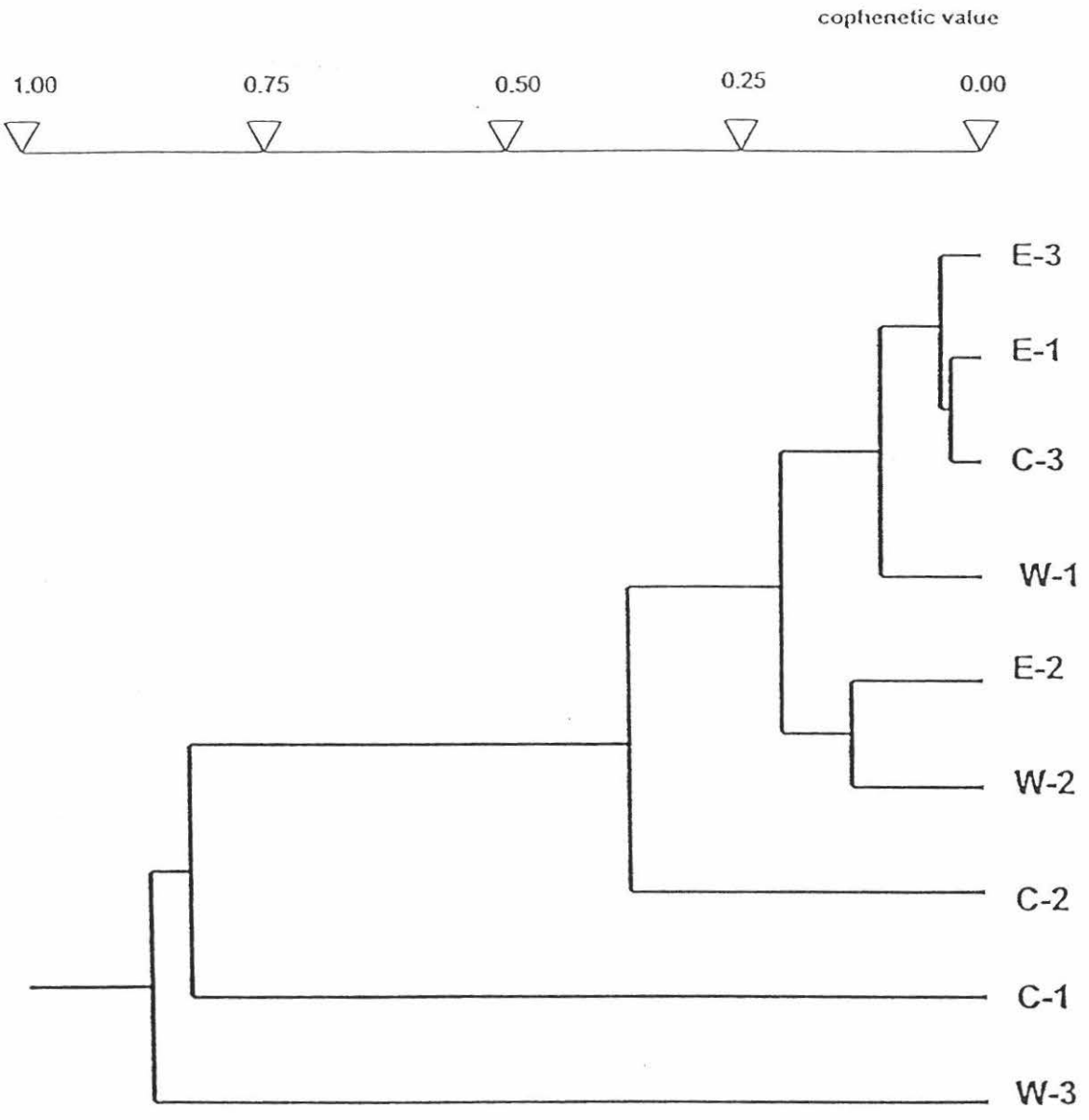

Figure 12. UPGMA Dendogram Based on Isozymes for the Populations of Sarotherodon mossambicus Under Study.

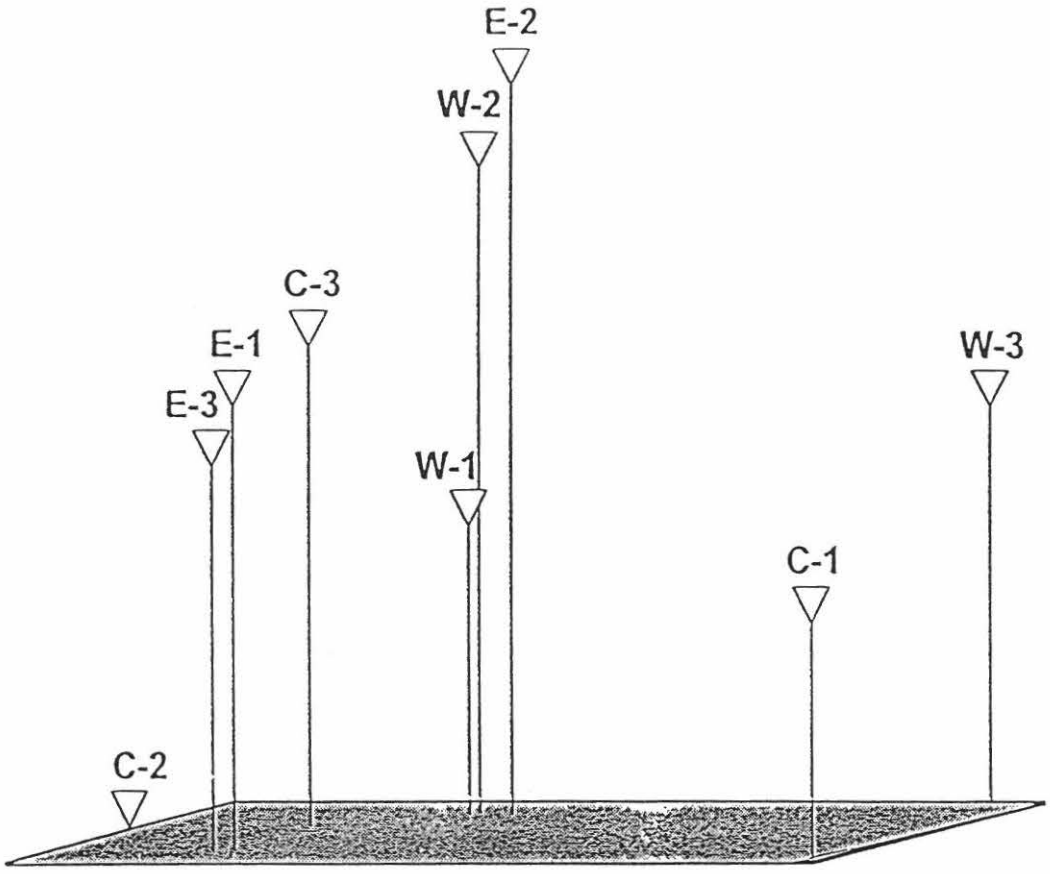

Figure 13. PCO Diagram Based on Isozyme for the Populations of Sarotherodon mossambicus Under Study.

Table 16. Exact Tests of Isozyme Polymorphic Loci for *S. mossambicus* Populations Under Study [1)]

Locus	Populations								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
Gpi-1	ns	-	-	-	ns	ns	ns	-	ns
Gpi-2	ns	-	-	-	ns	ns	ns	-	ns
Es-3	-	**	ns	ns	ns	ns	ns	ns	ns
Es-4	ns	ns	ns	ns	ns	ns	ns	ns	ns
Me-1	ns	ns	ns	ns	ns	ns	ns	ns	ns
Me-2	ns	ns	ns	ns	ns	ns	ns	ns	ns
Aco	-	*	*	-	ns	-	-	-	-
Ldh-3	-	-	**	-	-	-	-	-	-
Idh	**	-	-	-	**	ns	**	-	**
Fum	ns	-	ns	ns	-	-	-	-	-
Adh	-	-	-	**	-	-	-	-	-
Pgd	**	**	**	**	**	**	**	**	**

note: 1) - Locus fixed to a certain allele
* significant at 5%, no heterozygotes observed
** significant at 1%, no heterozygotes observed

were 67 tests conducted, out of which 18 deviated significantly from Hardy-Weinberg expectations. In the inbreeding test, an F_{is} values of 1.00 or more indicates significant deviation from Hardy-Weinberg equilibrium. Among these populations, however, none of the F_{is} coefficients reached this level (Table 17), indicating no significant deviations from Hardy-Weinberg expectations.

Other indices that may show genetic differences among the populations are shown in Table 18. They are the percent polymorphic loci (P), number of alleles per locus (A) and number of loci per polymorphic loci (A_p). Those statistics also indicate that the populations of T. mossambicus in this study were differentiated.

DISCUSSION

It appears that the S. mosambicus community in Java is significantly structured into more or less isolated populations with little gene flow. The genetic variation among the 9 populations studied accounted for 6.7 to 8.4% of the total gene diversity (G_{st}=0.067 for the unbiased calculation or G_{st}=0.084 for the unmodified calculation).

Table 17. Inbreeding Coefficients Based on Isozymes for Each Locus in the Populations of S. mossambicus Under Study [1)]

Locus	Populations								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
Gpi-1	-0.02	–	–	–	0.06	-0.02	-0.02	–	-0.02
Gpi-2	-0.02	–	–	–	0.06	-0.02	-0.02	–	-0.02
Es-3	–	–	-0.09	-0.04	-0.09	-0.09	-0.39	-0.09	-0.02
Es-4	0.38	-0.22	-0.14	0.34	-0.25	0.38	0.38	0.31	-0.39
Me-1	0.32	-0.06	0.36	-0.09	0.25	-0.04	-0.09	0.33	-0.04
Me-2	0.32	-0.14	0.32	-0.09	0.11	-0.04	-0.09	0.32	-0.06
Aco	–	–	–	–	0.11	–	–	–	–
Ldh-3	–	–	–	–	–	–	–	–	–
Idh	–	–	–	–	–	-0.04	–	–	–
Fum	-0.02	–	-0.09	-0.02	–	–	–	–	–
Adh	–	–	–	–	–	–	–	–	–
Pgd	–	–	–	–	–	–	–	–	–
F_{is}	0.284	-0.163	0.142	0.133	-0.013	0.160	0.121	0.261	-0.067
F_{is}'	0.515**	0.464*	0.478*	0.471*	0.214	0.226	0.455*	0.570**	0.511*

note: 1) " – " denotes F_{is}= 1.0 , the population is fixed at a certain allele.

F_{is} is calculated based on variable loci in each population, while F_{is}' is calculated based on the 12 polymorphic loci.

Table 18. Other Indices of Isozyme Gene Diversity Among *S. mossambicus* Populations Under Study

Pop.	H_{obs}	P	Number of alleles	
			A	A_p
W-1	0.030	29.63	1.30	2.00
W-2	0.027	22.22	1.26	2.17
W-3	0.037	29.63	1.33	2.13
C-1	0.027	25.93	1.26	2.00
C-2	0.055	33.33	1.33	2.00
C-3	0.028	29.63	1.33	2.13
E-1	0.033	29.63	1.33	2.13
E-2	0.030	18.52	1.19	2.00
E-3	0.019	29.63	1.33	2.13

note: 1) H_{obs} = observed heterozygosity
P = percent of polymorphic loci
A = number of alleles per locus
A_p = number of alleles per polymorphic locus

Many authors have studied genetic differentiation among populations of various fish species and have shown low, moderate and high levels of population division. Garcia-Marin et al. (1991) found a G_{st} value of 0.028 among hatchery populations of brown trout in Spain. This low level of differentiation indicated that the independently reared strains derived from the same broodstock, i.e. that the different hatchery populations had a common origin. In many cases, a low level of genetic divergence also suggests that gene flow among the populations is high. Such examples are among populations of Atlantic salmon in Scotland and Ireland with G_{st} value of 0.023 and 0.043, respectively (Jordan et al., 1992); among populations of galaxiids in New Zealand with G_{st}=0.017 (Allibone and Wallis, 1993); between brackish and freshwater populations of fourhorn sculpin in Scandinavia with G_{st}=0.013 (Gyllensten and Ryman, 1988); and among populations of marine species such as herring and plaice with G_{st} values of 0.012 and 0.004, respectively (Crozier and Ferguson, 1986). They also found moderate levels of genetic differentiation among brown trout populations in Lough Neagh catchments, Northern Ireland with G_{st} values ranging from 0.062 to 0.078; and in several other populations with G_{st} values of 0.037 to 0.111.

Examples of high levels of genetic differentiation occurred among non-diadromous species of galaxiids in New Zealand, with G_{st} values of 0.210 to 0.780 (Allibone and Wallis, 1993); and among natural populations of brown trout in Spain with G_{st} =0.615 (Garcia-Marin et al., 1991). Other studies of brown trout in a variety of habitats also demonstrated the ability of this species to become locally differentiated with high values of G_{st} ranging from 0.270 to 0.310 (Ryman, 1983). Oosthuizen et al. (1993) also found a high G_{st} value of 0.635 when studying the genetic distances and evolutionary relationships among 3 congeneric species of tilapia.

From the above investigations, the G_{st} value of 0.067 to 0.085 from the populations of S. mossambicus in Java can be considered as moderate, suggesting an intermediate level of genetic divergence among the populations. This G_{st} value also indicates that a moderate level of adaptive differences has independently evolved among those populations.

Statistically significant allele frequency differences were obtained among the 9 populations studied (χ^2=384.02; df=104; $p \leq 0.01$), suggesting significant differentiation among the populations. Another indicator of this divergence is the significant G_{st} value for unbiased calculation (χ^2=30.15; df=8; $p \leq 0.01$) or F_{st} value for unmodified

calculation (χ^2=37.80; df=8; $p \leq 0.01$). The overall differences among the populations resulted in a mean Nei's genetic distance of 0.005, ranging from 0.000 to 0.023.

Interspecific and intraspecific Nei's genetic distances have also been calculated among various populations of fishes. According to Shaklee et al. (1982), an average genetic distance of 0.050 (ranging from 0.002 to 0.065), 0.300 (ranging from 0.025 to 0.609) and 0.900 (ranging from 0.580 to 1.210) occur between conspecific populations, congeneric species and confamilial genera of fishes, respectively. Grant (1987) found that the average genetic distance between Atlantic and Pacific salmon was 0.264 which was typical for species level divergence, although the American Fisheries Society considered them as subspecies due to their morphological similarities. Two distinct geographic races were also found among the populations of salmon in the North Pacific, having an average genetic distance from one another of 0.039. The genetic distance among populations in each race averaged 0.003. Intraspecific genetic distances ranging from 0.006 to 0.016 were observed among populations of brown trout in Northern Ireland, with an estimated time of divergence from 8,000 to 79,000 years ago (Crozier and Ferguson, 1986). Low genetic distance values of up to 0.010 occurred among diadromous

populations of galaxiids in New Zealand. On the other hand, higher values of intraspecific genetic distance were found among non-diadromous populations ranging from 0.007 to 0.368 (Allibone and Wallis, 1993). Interspecific genetic distances of 0.070 to 0.100 were found between 2 species of tilapia, T. guisana and T. sparmanii. These 2 species are considered to be close relatives that diverged about 1.19 my ago. Genetic distance values ranging from 0.510 to 0.760 were obtained between these 2 species and a more distantly related species, T. rendalli (Oosthuizen et al., 1993).

Considering the above findings, the average genetic distance of 0.005 calculated from the populations of tilapia in this study was within the intraspecific range, and are typical for genetic distances of conspecific populations. According to Chakraborty and Nei (1977) genetic distance is strongly influenced by the bottleneck effect and that distance rapidly increases during the early generations after the bottleneck. Nevertheless, the effect depends on the average heterozygosity of the original population and on the bottleneck size. If the bottleneck size is not small or in some cases the population grows rapidly after a small bottleneck, the bottleneck effect remains relatively small. The bottleneck effect disappears when the average heterozygosity in the population is restored.

The amount of time since taxa shared a common ancestor can also be estimated from genetic distance value (Grant et al., 1988). Compared with estimates of divergence time based on geological data, a genetic distance value of 1.000 between fish taxa is considered to be equivalent to 18 million years of divergence time (Gorman et al., 1976; Vawater et al., 1980; Grant, 1987). Using this time scale, the separation of S. mossambicus populations in Java that now have an average genetic distance of 0.005 would have occurred some 90,000 years ago. However, according to Vass and Hofstede (1952) and Pullin (1988), S. mossambicus became established in Java in about 1938 when a fishery worker (Mr. Mujair) discovered 2 females and 3 males of this species. Two conclusions can be drawn from my study regarding the history of S. mossambicus populations in Java. First, assuming that the reported history from the literature is correct, then the rate of population differentiation among S. mossambicus populations in Java has been extraordinarily rapid. Thus, within a period of 55 years, the populations have reached an average distance value of ideal populations that have diverged for 90,000 years. Evidence for rapid differentiation during the early stage after populations experience a severe bottleneck has been well modeled (Selander and Whittam, 1983; Chakraborty and Nei, 1977). My

study provides an example of real rates under the stated and presumed conditions which is more than 1800 times faster than expected under Hardy-Weinberg conditions. Secondly, it is possible that the reported history of S. mossambicus populations in Java from the literature is not correct and that tilapia were introduced to Java at a much earlier date. However, the rate of growth for these fishes is so rapid that it is unlikely that they would have existed in small enough population to go undetected for many years.

The pattern of divergence among the 9 populations of tilapia in this study is visualized through UPGMA and PCO analyses (Figures 12 and 13). In general, population C-3, E-1, E-2, E-3, W-1 and W-2 were not genetically distinguishable. Among these populations, the distribution of gene frequencies for 9 out of the 12 polymorphic loci (GPI-1; GPI-2; ES-3; ME-1; ME-2; ACO; LDH-3; FUM and ADH) were not significantly different as shown from the pattern of differentiation in Table 14. Three loci, ES-4, IDH and PGD may have caused the given clustering pattern among these populations. Regarding the gene frequency distributions of ES-4, population E-3 was different from population C-3, E-1, W-1 and W-2, whereas population C-3, E-1 and W-1 were different from population W-2. With IDH locus, population E-1, E-3 and W-1 were different from population E-2 and W-2.

With the 3-allelic PGD locus, each population showed a unique gene frequency distributions. Population C-1, C-2 and W-3 were individually separated, indicating the existence of unique allelic combinations within each of them. Population C-2 had the relatively highest frequency of allele A from the ACO locus. In fact, heterozygous individuals regarding this locus were only found in population C-2, suggesting that heterozygous individuals for ACO were relatively more common in population C-2. Population C-1 had a private allele A of ADH locus at a frequency of 0.12, while population W-3 had a private allele A of LDH-3 locus at a frequency of 0.16.

The clustering pattern revealed from both UPGMA and PCO analyses did not show a correspondence between geographic distance and genetic identity. According to Crozier and Ferguson (1986), such a pattern of genetic relationship reflects local selective adaptations of non-neutral loci and/or random divergence of neutral loci in the absence of gene flow. Local selection and random genetic drift which also cause population divergence are discussed below.

According to Chakraborty and Leimar (1987), Grant (1987), Hartl and Clark (1989) and May and Krueger (1990), genetic differentiation among intraspecific populations evolves if migration or genetic flow is absent or small

compared to the forces that cause the differentiation. Those forces are mutation, genetic drift (or effective population size) and selection.

In this case mutation does not seem to be a likely cause of the observed intraspecific differentiation, because the rate of mutation creating non-lethal new alleles is quite low. Additionally, the time required for the spread of any new alleles over the species range by simple gene flow is much longer than the putative time since the populations diverged . However, unique alleles may be detected in some populations as the result from loss of alternative alleles by drift or selection after long term isolation. As already discussed earlier, significant differentiation exists among the populations of S. mossambicus in Java. Since this differentiation is unlikely to be due to mutation, the unique alleles detected in population C-1 (allele A of ADH locus) and in population W-3 (allele A of LDH-3 locus) are therefore more likely to be the result of ongoing drift and/or selection.

Genetic drift may be the main factor causing the differentiation among the populations of tilapia in this study through multiple founder events. According to Vaas and Hofstede (1952), S. mossambica was first discovered in the south coast of East Java at the mouth of the river

Serang in Blitar in 1939. Before this time, tilapia and other cichlid fishes were not known in Java nor in Indonesia. S. mossambica was then introduced and distributed throughout the island of Java for culture. The number of founders during the transfers were reported to ranged from 29 to 50 individuals. A number of these founders were lost or did not survive before they propagated. Therefore, the effective population size of those founders were often smaller than 29 individuals. Hedgecock and Sly (1990) also stated that population bottlenecks, such as the founding of a stock with a few wild broodstock, often have a great effect on allelic diversity. In contrast to selection, which differentially affects certain genes, genetic drift potentially affects all polymorphic loci in the same way.

Selection pressure from the varying local conditions results in adaptation to the local environment. If the selective differences between alleles of a locus are small, genetic drift becomes the main factor that causes differences among the populations. Chakraborty and Leimar (1987) further state that if populations have been sufficiently isolated to allow allele frequencies to drift apart and the separation is maintained, local adaptation is to be expected as well.

Quantitative analysis of the relative contribution of drift and selection to changes in gene frequencies of hatchery populations have recently been made (Gaffrey et al., 1992; Hedgecock and Sly, 1990; Vrijenhoek et al.,1990). For natural populations, however, the relative contribution of drift and selection to the gene frequency variation observed from protein electrophoresis is not yet resolved (Koehn et al., 1983; Nei, 1983). In this study, the existence of local selection apparently occurred at the ES-4 locus. Looking at the gene frequency distribution of this locus, population W-2 favors allele A, while the other populations seem to select allele B. In this case, selection force may be stronger than genetic drift in differentiating population W-2 from the others (The detail of this process is not covered in this present study). For the other polymorphic loci, local selection pressure seemed to be similar across the populations as the trend of gene frequency distributions was similar across those populations. For these loci, it is possible that genetic drift, not local selection was the stronger force causing the differences among the populations.

Significant deviations from the Hardy-Weinberg expected genotypic frequencies were obtained in some tests, because heterozygous individuals were not observed in those

particular loci (Table 8). All loci that deviated from the Hardy-Weinberg expectation had an F_{is} value of 1.00 (Table 9). This deficiency of heterozygous individuals can be due to inbreeding, selection or genetic drift. However, according to Hartl and Clark (1989), except for plants that have a high frequency of self fertilization or for certain insects that regularly undergo parent-offspring or brother-sister mating, the values of F_{is} in most natural populations are typically close to zero. From this statement, therefore, the unity values of F_{is} occurring in the populations of tilapia in this study were more likely due to genetic drift and selection. In general, when the F_{is} for each population was computed from variable loci only (Table 9), the genotypic frequencies in all 9 populations did not deviate from the Hardy-Weinberg expectation.

Hedgecock and Sly (1990) and Jordan et al. (1992) also mentioned that genetic drift can reduce the genetic diversity in a population. The drift is accentuated if a bottleneck occurs in that population. The genetic diversity of each population of tilapia measured by the observed heterozygosity varied from 0.019 to 0.055 in this study. The past bottleneck seemed to take the major factor in determining the heterozygosity within each of the populations. The bottleneck occurred to each population due

to the small initial population, an effect magnified by predation by aquatic carnivorous animals and unfavourable climate during the early period after introduction. The climate in East Java particularly is characterized by periodically long and dry monsoon season causing the shortage of water and a challenge to keep the breeders alive until the following wet season (Vaas and Hofstede, 1952).

With assumptions of no migration and the same genetic processes after introduction to each site, the history of S. mossambicus distribution in Java can be roughly inferred from the dendogram and heterozygosity (observed-H). Population C-2 had the highest observed-H, therefore population C-2 can be tentatively identified as the putative initial population of tilapia in Java. Some founders were transferred from C-2 to C-1, W-3 and E-2. Population E-2 then became the source of founders for population W-1, W-2, C-3, E-1 and E-3. However, this biogeographical history is not consistent with anecdotal accounts in which S. mossambicus are said to have been first found in Java at locations closest to E-1 in 1939 (Vaas and Hofstede ,1952). Some individuals were taken from E-1 and transferred to a location near E-2. From E-2 some founders were given to a research station in Bogor in 1941, a location near to W-2. A farmer in Tasikmalaya, a location very near to W-2, also

received some founders from E-2. In 1942 to 1946, S. mossambicus was extensively distributed in Java for farming for the purpose of becoming a main protein source for the community.

The biogeography of tilapia in Java inferred from allozyme data does not match well with the reported history from the literature. The most probable reason is that there was gene flow among the populations due to human intervention during and after transfers. Even a small amount of gene flow can greatly disrupt genetic drift (Hartl and Clark, 1989), and thus affect the observed-H.

Regardless of the biogeographical history of S. mossambicus in Java, significant population differentiation of this species appears to have occurred in this island, putatively in less than 60 years.

CHAPTER FOUR

RAPD ANALYSIS OF S. mossambicus POPULATIONS IN JAVA

INTRODUCTION

Molecular techniques have provided powerful tools not only for estimating genetic diversity within and among populations, but also for analysis of relationships between organisms. Isozyme and allozyme methods used in population genetic studies are cost effective, but many times the paucity of loci exposed and the markers obtained through this method restrict their usefulness for some purposes such as breeding programs and population studies (Black IV et al., 1992; Ragot and Hoisington, 1993; Puterka et al., 1993; Heun et al., 1994). Therefore, genetic data at the DNA level obtained from RFLPs, hybridization, DNA fingerprinting and nucleic acid sequencing are increasingly preferred as those data are frequency and distance data and are relatively more informative. However, these conventional DNA techniques are laborious and relatively complicated, requiring special training. They are costly and often require relatively greater amounts of DNA per sample (Hadrys et al., 1992). The need for large amounts of DNA per sample

was eliminated after Kery Mullis developed the polymerase chain reaction (PCR) as an effective *in vitro* cloning method in the late 1980s. But PCR brings another obstacle because this technique requires knowledge of the target DNA sequence for primer design.

Recently, another DNA technique called Random Amplified Polymorphic DNA (RAPD) was introduced by Williams *et al.* (1990). This method is capable of producing many useful nuclear markers (RAPD markers). Different from the ordinary PCR procedure that uses 2 primers, the RAPD technique amplifies segments of genomic DNA with a single primer of arbitrary nucleotide sequence. That single primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance from each other, i.e. from 50-200 to 3000-4000 bp (Williams *et al.*, 1993; Johnson *et al.*, 1993; Van Coppenolle *et al.*, 1993). The standard RAPD method uses arbitrarily synthesized primers 9-10 nucleotides long, containing 50-80% G+C without palindromic sequences. A selected primer used in this method will randomly bind to many sites within the genome. There is a high probability that the genome contains several priming sites close to each other that are in an inverted orientation. The primer that binds to those small inverted repeats amplifies the intervening DNA segments of variable length (Hadrys *et al.*,

1992). Some primers amplify up to 29 segments of genomic DNA during one PCR amplification program (Huff et al., 1993; Riedy et al., 1992). However, some segments are amplified in 1 individual but not in another due to absence of or mutation within a primer binding site. Therefore DNA polymorphisms can be detected from the amplification products of different individuals.

The RAPD products are separated in 1.4-2.0% agarose gels and visualized by Ethidium-bromide staining. A negative reaction or control for each primer, composed of a preparation without a target DNA sample, is necessary to confirm that the observed bands are the amplified genomic DNA and not primer artifacts or other contaminants. Each RAPD band is then considered as a locus, therefore, the number of loci that can be generated from RAPD methodology is essentially unlimited.

In order to validate the statistical analysis of RAPD data, several assumptions are made (Lynch and Milligan, 1994): a) each locus can be treated as a 2 allele system and only 1 allele (the dominant or the marker allele) is amplified; b) marker alleles from different loci do not comigrate to the same position on a gel; and c) the investigator is fully capable of matching bands from different lanes within and among gels. Regarding the first

assumption, Williams et al. (1990) and Hedrick (1992) stated that the exact basis of the polymorphisms has not been understood yet. When the recessive or null allele fails to amplify, it can be due to variation in sequence, to a deletion that causes the loss of the primer site, or to an insertion that changes the size of the DNA segment with or without preventing its amplification.

The criticisms of the RAPD method deal with the first and second assumption mentioned above (Lynch and Milligan, 1994). The dominant nature (presence or absence) of RAPD markers renders this technique incapable of showing heterozygote individuals directly and this characteristic reduces the accuracy of gene frequency estimation. Comigration of bands is also of concern because the RAPD technique cannot distinguish the products of different loci that have similar molecular weights. Therefore, data from RAPD techniques tend to violate the assumptions to some extent. Another concern is about the repeatability of the banding pattern that may at times be difficult to obtain (Hedrick, 1992).

Some of these problems concerning RAPD method can be overcome. Hadrys et al. (1992) gave a practical means to reduce comigration by using polyacrilamide gel electrophoresis to increase the resolution of band

separation, or by eluting individual PCR bands from gels and reprobing the products through Southern analysis. Hedrick (1992) suggested analyzing many more loci to increase the statistical power, in order to reduce the effect of dominance. According to Lynch and Milligan (1994), to achieve the same degree of statistical power using codominant markers, the sample size per locus should be in the order of 2-10 times with RAPDs. Moreover, the frequency of the marker allele in the selected loci for analysis should be relatively low, i.e. less than 1 - 3/n; or, the frequency of the null allele is relatively high. Concerning the reproducibility of the banding patterns, Hadrys et al. (1992) and Wolff et al. (1993) stated that it can be achieved by optimizing the PCR conditions. According to Yu and Pauls (1992) and Adams and Demeke (1993) those conditions include the duration and temperature of the PCR operation and the concentrations of the PCR reactants, i.e. the concentration of polymerase enzyme, magnesium chloride, primer, dNTPs, buffer and DNA sample.

The RAPD method has several advantages compared to the other DNA techniques. RAPD bands can be easily separated and visualized on standard agarose gels with ethidium bromide staining, without any radiolabeled probes. Besides its convenience and relative simplicity to conduct, the RAPD

method is also cost and time effective, requiring small amounts of DNA per sample (1-10 microgram), performing random selection of sites within the genome and having a broad range of applications (Williams et al, 1990; Hedrick, 1992; Ragot and Hoisington, 1993).

Continuous efforts are being made to refine the RAPD method. In spite of its weaknesses, the technique has been used successfully to reveal the genetic properties of various organisms in various fields of study. Application of RAPDs for constructing genetic maps has been done in soybean (Williams et al., 1993), eucalyptus (Grattapoglia and Sederoff, 1994) and zebra fish (Postlethwait et al., 1994). Population studies have also been conducted using RAPDs in buffalo grass (Huff et al., 1993), wild oat (Heun et al, 1994), wheat (He et al., 1992; King et al., 1993), flowering plants (Fritsch et al., 1993), fungus (Meijer et al., 1994), aphid (Black IV et al., 1992), nematode (Hahn et al., 1994), and fish (Johnson et al., 1994). Many other studies using RAPDs have been documented, such as in systematics and phylogenetics of Juniperus (Adams and Demeke, 1993), Azolla (Van Coppenolle et al., 1993) and aphids (Puterka et al., 1993); in taxonomic identification and fingerprinting of tilapia fish (Bardakci and Skibinski, 1994), rice and microbes (Welsh and McClelland, 1990;

Jayarao et al., 1992); in parentage and relatedness of primates (Riedy et al., 1992), hydrozoans (Levitan and Grosberg, 1993), beetles (Scott et al., 1992), alfalfa (Yu and Pauls, 1993) and cereals (Dweikat et al., 1993); hybridization and introgression of Yucca (Hanson, 1993) and iris (Hadrys et al., 1992); linkage marker of the downy mildew resistance gene in lettuce (Paran and Michelmore, 1993) and the supernodulation locus in soybeans (Caetano-Analles et al., 1993); and in the generation of probes for some microorganisms (Fani et al., 1993).

The use of RAPDs for fishery studies so far is still at the early stage (Dinesh et al., 1993; Postlethwait et al., 1994; Bardakci and Skibinski, 1994). The analysis of RAPD data in this study is therefore conducted as a comparison to isozymes in order to know the effectiveness and utility of RAPD markers in fishery population genetic studies.

MATERIALS AND METHODS

Sample Collection

The same individuals of S. mossambicus used for morphometric and isozyme analysis were used in the RAPD analysis. Heart tissue was taken from each individual

before the fishes were treated for morphometric data. The tissue was put in a 1.5 ml microfuge tube and kept in dry ice during collection and transport, and then transferred into a -86°C freezer upon arrival at the laboratory (Baverstock and Moritz, 1990).

DNA was extracted from the heart tissue following the salting-out procedure of Miller et al. (1988). The procedure was slightly modified to fit a mini preparation extraction in a 1.5 ml tubes (Appendix 5). The extracted DNA was dissolved in TE buffer pH 7.5 (Table 19); the quality of the DNA was examined by 0.9% agarose gel electrophoresis coupled with ethidium bromide visualization.

Ten DNA samples were selected from each population, and then the DNA was quantified using a Hoefer TKO-100 fluorometer. The concentration of the working solution of DNA was made equal to 10 ng/μl for all samples. The stock DNA solutions were kept at -20°C, while the working solutions were put in a 4°C refrigerator.

Data Collection

The amplification of DNA was performed in a MJ-thermocycler, then the PCR products were examined by 2% agarose gel electrophoresis and ethidium-bromide staining. The data collection consisted of 2 stages, the survey stage

Table 19. PCR-Progams Used in the Study [1]

Steps	Kocker et al. (1989)	Williams et al. (1990)	Selected
I	2 minutes; 93°C	1 minute ; 94°C	1 minute ; 94°C
II	1 minute ; 93°C	1 minute ; 94°C	1 minute ; 94°C
III	1 minute ; 50°C	1 minute ; 36°C	1 minute ; 39°C
IV	2 minutes; 72°C	2 minutes; 72°C	3 minutes; 72°C
V	40 x from II	45 x from II	45 x from II
VI	7 minutes; 72°C	7 minutes; 72°C	7 minutes; 72°C
VII	58 hours ; 4°C	58 hours ; 4°C	72 hours ; 4°C
VIII	end	end	end

note: 1) 'selected' is the modification of Williams et al. (1990) used in the main step of this study.

sample DNA was dissolved in TE-buffer pH 7.5 (10 micro-L of 1M Tris + 100 micro-L of 2mM Na_2-EDTA + 890 micro-L of dH_2O, adjusted to pH 7.5).

and the main stage; the survey stage was intended to determine the optimum protocol in terms of the PCR program and PCR reaction.

Two PCR programs were tested (Table 19); one program is by Kocher et al. (1989) and the other is by Williams et al. (1990). Modification of the second program on the annealing temperature (Step 3) and extension time (Step 4) were also tried. Based on the quality of the products, the second program was selected with a modified annealing temperature of 39°C and extension time of 3 minutes (Table 19).

Optimization of the PCR reaction was done on the concentrations of magnesium-chloride, dNTPs, polymerase enzyme (Taq) and DNA sample. The concentrations tested and then selected are shown in Table 20. The decisions were made based on the number of clear and reproduceable bands generated.

The survey stage was finalized by performing primer selection. Eighty decamers of Operon Technologies, Inc., namely decamers of their Kit A, Kit B, Kit C and Kit D, were tested in order to find primers that give many polymorphic loci or bands. The primers giving the most bands (Table 21) were then used in the primary PCR experiment.

In the primary study, each selected primer was run on a total of 90 DNA samples from the 9 populations (10 samples

Table 20. Concentrations of PCR-Reactants Used in the Preliminary and Primary Study [1)]

$MgCl_2$ (μ-L)	dNTP's (μ-L)	Polymerase (μ-L)	DNA-sample (μ-L)
1.5	0.5	0.10	0.5
2.0	1.0	0.13	1.0
2.5			1.5
3.0			
3.5			
3.0*	1.0*	0.13*	1.0*

note: 1) stock $MgCl_2$ is 25 mM

stock dNTP's is 10 mM (2.5 mM for each dNTP); from Epicentre Technologies, Inc.

stock Taq DNA-Polymerase is 5 units per μ-L; from Promega with Catalog # M1861

working solution of DNA-sample is 10 ng/μ-L.

*) amount used in the primary stage of this study.

Table 21. Selected Decamers Used in the Primary Study [1)]

Primer Code	5' to 3' Nucleotide Sequence	polymorphic markers
A-18	AGGTGACCGT	2
A-20	GTTGCGATCC	3
B-01	GTTTCGCTCC	1
B-03	CATCCCCCTG	9
B-04	TGCGCCCTTC	4
B-05	CTGCTGGGAC	5
B-10	GGACTGGAGT	1
B-17	AGGGAACGAG	1
C-02	GTGAGGCGTC	5
C-04	CCGCATCTAC	4
C-07	GTCCCGACGA	7
C-09	CTCACCGTCC	3
C-13	AAGCCTCGTC	1
C-14	TGCGTGCTTG	1
D-03	GTCGCGGTCA	1
D-05	TGAGCGGACA	2
D-20	ACCCGGTCAC	1
Total of 17 primers		Total of 51 markers

note: 1) The decamers were of RAPD-Primer Kits from Operon Technologies, Inc.;
15 μ-g of each lyophylized decamer was dissolved in 1000 μ-L TE-buffer pH 7.5 to make the working solution of 15 ng/μ-L;
The TE-buffer was made of 10 μ-L of 1 M Tris, 100 μ-L of 2 mM Na_2-EDTA and 890 μ-L of dH_2O, adjusted to pH 7.5 .

per population), using the mix of reactants listed in Table 22. After each run, the PCR products were visualy examined, photo-documented and scored. Scoring of each polymorphic locus was done by noting presence of a band as (+) and the absence of a band as (-). Assuming that Hardy-Weinberg equilibrium is achieved within each population (which is justified in this case by isozyme analysis), the gene frequency of the marker (dominant) allele and the null (recessive) allele were estimated for each locus. The genotype of individuals with absent bands was considered as homozygous recessive. The proportion of these individuals within each population is therefore equal to $(q)^2$, where q is the frequency of the null allele:

q = proportion of homozygous recessive in sample

and the frequency of the marker allele is:

$p = 1 - q$

Data Analysis

The gene frequency data were analyzed for the Nei's coefficient of genetic differentiation G_{st} and standard genetic distance using the GENESTAT-PC program version 3.3 of Lewis (1993). The chi-square tests of significance of the population differentiation were done according to Yoshiyama and Sassaman (1983).

Table 22. The Composition of 25 μ-L PCR-reaction Used in the Primary Study

Substance	Amount (μ-L)
$MgCl_2$ of 25 mM	3.00
dNTP's of 10 mM	1.00
Reaction Buffer *)	2.50
Taq-Polymerase of 5 u/μ-L	0.13
Primer of 15 ng/μ-L	1.00
DNA sample of 10 ng/μ-L	1.00
dH_2O	16.37

note: *) Reaction Buffer is a 10x buffer that contains 200 mM Tris/HCl pH 8.0 and 500 mM KCl.

Analysis of Molecular Variance (AMOVA) according to Excoffier et al. (1992) and Huff et al. (1993) was also performed in order to ascertain the significance of the population differentiation.

Chi-square tests of gene frequency heterogeneity was then conducted for each locus using the microcomputer version of SYSTAT, in order to determine the loci that cause the differentiation.

The overall structure of population divergence was analyzed with UPGMA dendogram and Principal Coordinate Analysis (PCO analysis) using NTSYS-PC version 1.40 of Rohlf (1988).

RESULTS

Table 21 shows the selected decamers used in this study as well as the number of polymorphic loci produced by each decamer. Out of 17 selected decamers with 60-70% G+C content, 51 markers (or bands = loci) were generated; each decamer was able to produce 1 to 9 markers.

Gene frequencies of marker (dominant) and null (recessive) alleles for each locus for each population are given in Appendix 6. Analysis of the gene diversity

statistics resulted in significant values of G_{st}= 0.217, unbiased for sample size (χ^2=38.95; p<0.01) and G_{st}= 0.253 with unmodified data (χ^2=45.30; p<0.01). Those G_{st} values indicated a highly significant population differentiation among the whole community of S. mossambicus in this study. When tested for each locus, the differentiation was significant for 41 loci out of the 51. Table 23 shows the loci that significantly contribute to the overall differentiation.

The AMOVA in Table 24 also showed a highly significant population divergence among the community of tilapia in this study (Phi_{st}= 0.214; p<0.001). This AMOVA therefore confirms the analysis of gene diversity statistics above. The genetic variation among populations accounted for 21.35% of the total variation, while the within population variation accounted for 78.65% of the total variation.

The overall genetic differences between every pair of population comparison resulted in Nei's standard genetic distances ranging from 0.058 to 0.229 with a mean value of 0.136 (Table 25).

Finally, the pattern of the overall population structure is depicted by a UPGMA generated dendogram (Figure 14) and a PCO diagram (Figure 15). The dendogram clusters population E-1, E-2, E-3, C-3, W-2 and W-3

Table 23. RAPD Loci that Significantly Contribute to the Population Differentiation

Locus Code*	χ^2	Locus Code	χ^2
A18-1.15	50.16	C02-0.55	61.51
B01-1.15	64.13	C04-1.40	35.13
B03-2.50	33.03	C04-1.05	26.99
B03-1.65	38.27	C04-0.85	21.70
B03-1.55	39.83	C04-0.45	21.68
B03-1.50	34.70	C07-1.35	24.70
B03-1.20	61.39	C07-0.90	41.73
B03-0.85	44.64	C07-0.85	17.25
B03-0.75	33.32	C07-0.80	34.83
B04-1.95	75.38	C07-0.55	29.59
B04-1.85	34.67	C09-0.45	59.94
B05-2.30	81.83	C09-0.40	51.54
B05-1.50	85.76	C13-2.35	48.67
B05-1.00	29.29	C14-1.00	44.10
B05-0.95	80.95	D03-1.85	51.35
B05-0.85	99.34	D05-1.00	59.53
B10-0.70	55.61	D05-0.70	57.44
B17-1.65	61.57	A20-1.65	66.68
C02-2.50	71.04	A20-1.35	60.78
C02-0.70	23.42	A20-1.30	70.23
C02-0.60	102.65		

note: *) Locus code is primer code followed by fragment size in kb.

Table 24a. Gene Diversity Statistics Based on RAPD-Markers for S. mossambicus Populations Under Study [1)]

Data	H_s	D_{st}	H_t	$G_{st}= F_{st}$	χ^2
Unmodified	0.2817	0.0952	0.3769	0.2526	45.30**
Unbiased for Sample Size	0.2966	0.0822	0.3788	0.2172	38.95**

note: 1) $\chi^2 = 2N \times G_{st} = (2)(89.67)(G_{st}) = 179.34 \times G_{st}$; df = 8

$\chi^2_{8;0.05}= 15.5$; $\chi^2_{8;0.01}= 20.1$

Table 24b. Analysis of Molecular Variance (AMOVA)for 86 Individuals Using 51 RAPD Markers [2)]

Source	df	SSD	MSD	Variance	t-total	p-value
Population/Java	8	217.25	27.16	2.05	21.35	<0.001
Individuals/pops	77	582.05	7.56	7.56	78.65	<0.001
Total	85	799.30				

The $Phi_{st}= 0.214$

note: 2) 4 individuals are excluded due to missing values for some loci

Table 25. Nei's Standard Genetic Distances Based on RAPDs Among *S. mossambicus* Populations Under Study

Pop	Populations								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
W-1	–	–	–	–	–	–	–	–	–
W-2	0.058	–	–	–	–	–	–	–	–
W-3	0.076	0.078	–	–	–	–	–	–	–
C-1	0.156	0.112	0.128	–	–	–	–	–	–
C-2	0.167	0.140	0.123	0.088	–	–	–	–	–
C-3	0.217	0.172	0.124	0.125	0.061	–	–	–	–
E-1	0.099	0.105	0.103	0.142	0.111	0.119	–	–	–
E-2	0.145	0.167	0.108	0.170	0.140	0.136	0.113	–	–
E-3	0.146	0.226	0.163	0.299	0.247	0.216	0.187	0.091	–

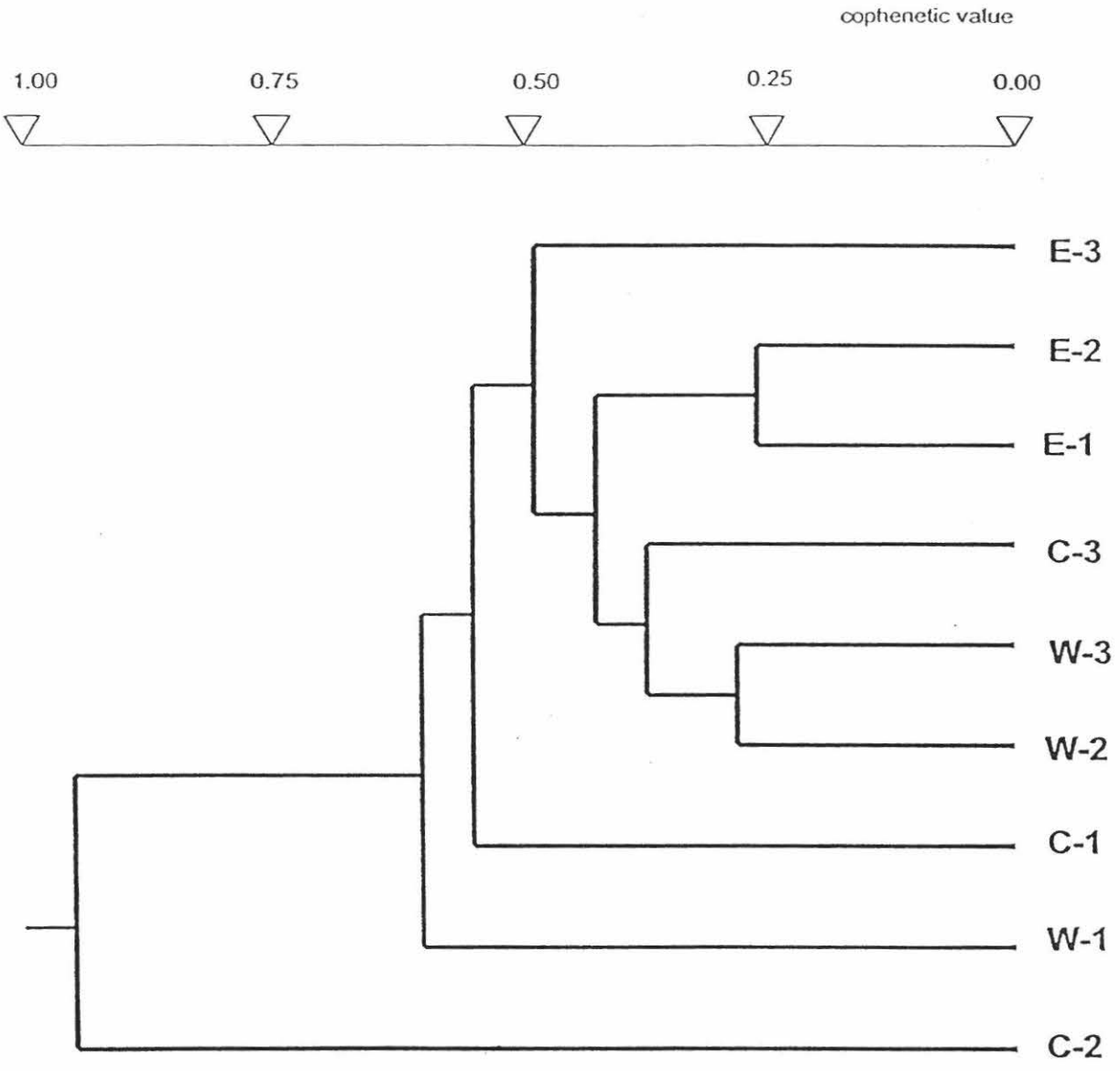

Figure 14. UPGMA Dendogram Based on RAPDs for the Populations of Sarotherodon mossambicus Under Study.

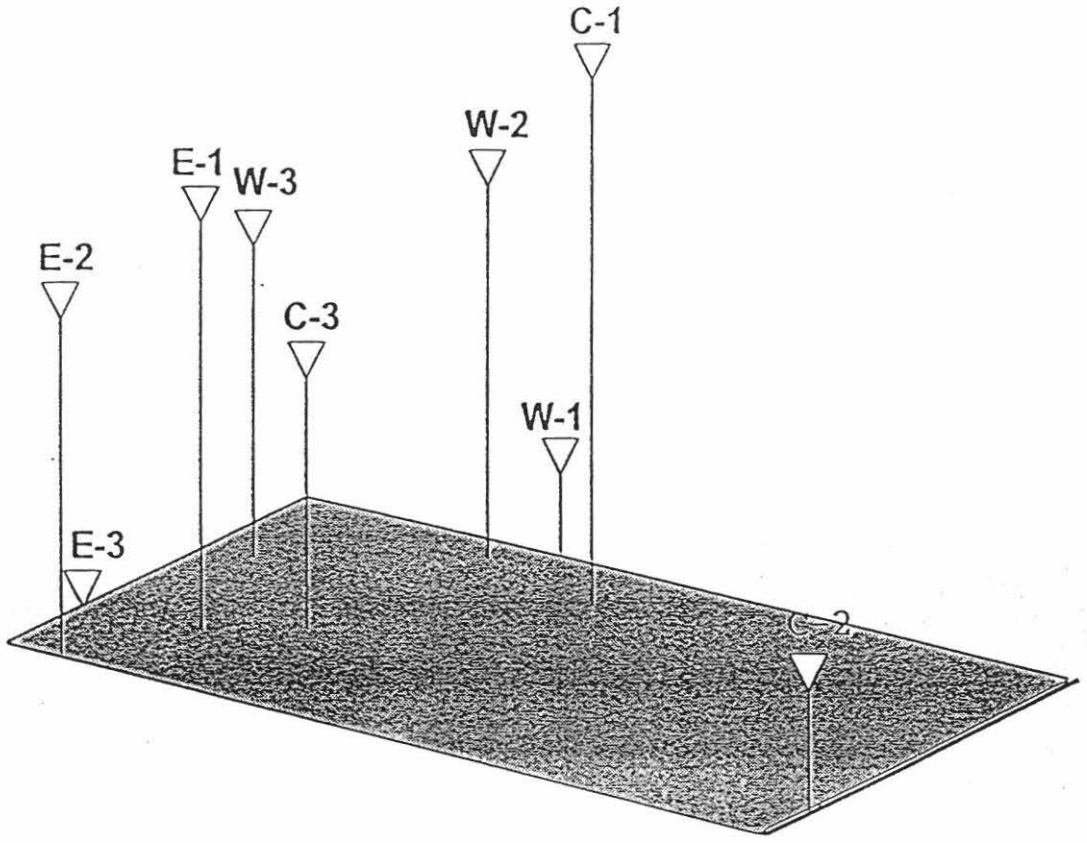

Figure 15. PCO Diagram Based on RAPDs for the Populations of Sarotherodon mossambicus Under Study.

together. Of these, population E-3 is the most different from the others. Population E-1 and E-2 are genetically close to each other, and so are population W-2 and W-3. Population C-3 is genetically closest to population W-2 and W-3. Population C-1, C-2 and W-1 are individually diverged. The PCO diagram yields a similar interpretation to the UPGMA dendogram. In this case, the 3 axes of the diagram, i.e. the 3 principal coordinates, together accounted for only 67.42% of the total genetic variation.

DISCUSSION

Highly significant population divergence was found among S. mossambicus populations in this study with RAPDs. The coefficient of genetic differentiation G_{st} of 0.217 and 0.253 are relatively high as compared to those obtained with isozymes which are only 0.067 and 0.085. These higher values suggest that the RAPD technique is a highly sensitive method for detecting population genetic differences. An increasing number of studies have been reported in which enzyme electrophoresis showed little or no variation, while RAPD data revealed substantial differentiation, such as among natural populations of fungal species (Meijer et al.,

1994), wheat aphid (Puterka et al., 1993; Black IV et al., 1992), aspen (Liu and Furnier, 1993), wild oat (Heun et al., 1994), and intraspecific fish populations of Oreochromis niloticus (Bardakci and Shibinski, 1994).

In this study, overall genetic distances among tilapia populations in Java were also relatively higher with RAPDs, ranging from 0.058 to 0.299 with an average of 0.136. The range of these distance values are consistent with differences found among congeneric species of fishes using isozyme data (Shaklee et al., 1982), and are comparable to the distance values observed among conspecific populations of Yucca baccata analyzed by Hanson (1993) using the same procedure that I have employed. Van Coppenolle et al. (1993) found that the genetic distance values among 3 congeneric species of Azolla based on RAPD data ranged from 0.760 to 0.880.

According to Van Coppenolle et al. (1993), the larger genetic distance values with RAPDs as compared to isozymes are due to greater polymorphisms that can be detected using the RAPD technique. Isozymes represent structural genes that make up only a small part of the genome, while the RAPD technique scans for polymorphisms within the total genome including highly variable as well as conserved regions of the genome (Black IV et al., 1992; Meijer et al., 1994).

This also means that the RAPD method measures more polymorphic sites than isozymes/gene products that are under natural selection. Johnson et al. (1993) found that the RAPD method could reveal extensive polymorphisms when comparing laboratory strains of zebra fish. Williams et al. (1993) also stated that the RAPD method is a powerful technique to survey polymorphisms in the genome, and that a single primer can be used to detect up to 100 loci by resolving the reaction products on a polyacrilamide gel and staining with silver. In this study, RAPD markers have effectively showed genetic substructuring among the populations of S. mossambicus in Java, eventhough these populations have been separated for less than 60 years. This indicates that in a short period of time a few individual founders have been able to recombine and outcross to produce detectable differentiation.

AMOVA analysis congruently demonstrates highly significant ($p<0.001$) genetic differences among the populations of tilapia in this study. Only 21.35% of the total diversity was attributable to these differences, the other 78.65% of the total genetic diversity was due to the variation among individuals within populations. In this study, each individual had a unique fingerprint for overall loci. Similar results were obtained by Huff et al. (1993)

when studying RAPD variation within and among natural populations of outcrossing buffalo grass. It is very likely that RAPD data can show extensive variation within populations; little or no RAPD variation within populations indicates an isogenic state or highly inbred nature of individuals within the populations. Huff et al. (1993) further stated that AMOVA is a powerful analysis that is capable of separating population differences against a background of a high level of polymorphisms within each population.

The overall population divergence is depicted in a UPGMA dendogram. Several similarities in the clustering pattern can be noticed between isozymes and RAPDs (Figure 12 and Figure 14). Both analyses show close relationships among populations E-1, E-2, E-3, C-3 and W-2. However, those populations are relatively more differentiated with RAPDs, one again indicating that the RAPDs technique is more powerful than isozymes in resolving population genetic differences in this study. The RAPD data placed population W-3 in the same cluster with population E-1, E-2, E-3, C-3 and W-2, while population C-1, C-2 and W-1 are individually diverged from the others. With isozymes, on the other hand, population W-1 is clustered together with population E-1, E-2, E-3, C-3 and W-2, while population W-3, C-1 and C-2 are

individually separated from the others. Similar results were found by Heun et al. (1994) when comparing RAPD and isozyme analysis for determining the genetic relationships among wild oat strains. The UPGMA dendogram with RAPD data gave more definitive separation of clusters of the strains. Puterka et al. (1993) also found that RAPD analysis, compared to isozymes, could show more genetic variations among aphid populations, and therefore a more informative dendograms could be constructed from RAPD data. A RAPD based dendogram seems to be more accurate for determining the relationships among populations which are too close to be differentiated by isozymes. Hence, isozyme based dendograms less adequately reflect the true pattern of genetic variation among populations.

The use of both UPGMA and PCO to analyze the data provides a better insight into population differentiation. Heun et al. (1994) and Van Coppenole et al. (1993) stated that the 2 methods weigh the data differently. UPGMA shows the minimum distances found in pairwise comparisons, whereas PCO gives a view of group relationships. Sneath and Sokal (1973) commented that the clustering method of UPGMA is best used for estimating relationships between close relatives but it poorly represents the relationships among clusters. Ordination methods such as PCO, on the other hand, are good

for showing the relationships between major groups but less sensitive to the relationships between close relatives. In this study, the overall relationships between populations of tilapia in Java were also analyzed using PCO. The first 3 axes (Figure 15) after PCO accounted for 67.42% of the total genetic variation among the 9 populations. With such percentages the diagram show some extent of consistency with the UPGMA diagram. Populations E-1 and E-2, and populations W-2 and W-3 are positioned close to each other. Populations W-2, W-3 and C-3 can be clustered together. Then, populations E-1, E-2, E-3, W-2, W-3 and C-3 can be made into one cluster, while populations W-1, C-1 and C-2 are individually separated.

Dinesh et al. (1993), after using RAPD markers for identification of 12 species of fishes representing seven families, stated that RAPD analysis is an efficient method of DNA fingerprinting in fishes. Bardakci and Skibinski (1994) have also successfully used the RAPD technique for species and subspecies identification of tilapia fishes. In this study, RAPD markers have been effectively used for population study of S. mossambicus in Java.

CHAPTER FIVE

GENERAL DISCUSSION

Genetic Documentation of *S. mossambicus* in Java

Identification of population using morphological data (with the method used in this study) can demonstrate population differentiation by size and shape. However, such documentation does not adequately provide information about the genetic background of a population, and therefore it may be highly transitory. Fish particularly are phenotypically more variable than other vertebrates. They can vary considerably in morphological features such as body size, growth rate, color and age of sexual maturity observed within as well as between populations, while showing little genetic variation (Allendorf et al., 1987; Sage and Selander, 1975; Turner and Grosse, 1980). Within this study, the patterns of differentiation among tilapia populations based on size and shape are inconsistent with those based on isozymes or RAPD. This suggests that size and shape differences are not primarily genetic but are most likely environmentally induced (Yoshiyama and Sassaman, 1983). Shape groupings are also inconsistent with groupings

derived from size measures. These results are consistent with models in which environmental conditions significantly affect growth and shape.

Shape and size differences among Javanese tilapia populations in this study are considered to be primarily nongenetic, since genetic data was not consistent with morphological data. This conclusion needs further confirmation, since such inconsistencies could alternately be obtained if sets of genes that control growth and development were not, by chance, sufficiently surveyed by isozyme/RAPD analysis.

The isozyme and DNA analysis revealed in my study, a significant genetic differentiation among the nine populations of S. mossambicus. These genetic data did not, however, provide a unique marker or set of diagnostic markers for any one of the nine populations. No markers were population specific, which is consistent with the belief that the nine populations are conspecific, even though there is significant population structuring among them. The genetic distance values as well as the G_{st} values derived from the gene diversity analysis (Table 24a and Table 25) support this conclusion. Additionally, the genetic distance values imply a rapid rate of population divergence.

This documentation study, has compared the utility of three methods for identifying population and measuring gene flow. The use of morphological characters for assessing genetic divergence can be misleading in 2 ways: a) morphological characters are thought to provide a large amount of genetic information because they are polygenic, when in fact a polygenic character with 100% heritability (no environment effect) contains only the same amount of genetic information given by a single electrophoretic locus; the information is greatly reduced if the heritability is less than 100%; b) large phenotypic variation coupled with the lack of information concerning the genetic basis for that variation often results in taxonomic oversplitting. This study suggests that environmental data should accompany morphological data especially when wild life management concerns are being addressed. The results of my study are consistent with the growing awareness that not all morphometric measures equally reveal relatedness and that morphological features need to be carefully studied in order to evaluate their usefulness in recognizing genetically distinct populations. Whenever possible, morphological documentation should be accompanied with biochemical data. For economical reasons, documentation at the protein level should be done first. Isozyme data may in itself give a

sufficiently broad picture of genetic variation within and among the populations. Isozyme analysis does not satisfactorily reveal genetic variation, in some cases and often requires the sacrifycing of the subject. If these conditions are limiting, the studies at the DNA level using RAPDs can provide sensitive, data-rich gels.

This study reveals genetic data that is consistent with theories of population differentiation through the founder principle (Templeton, 1980; Carson and Templeton, 1984; Leberg, 1992). Founder effects lead to changes in the genetic structure of the founder population. Changes usually lead to genetic divergence between the ancestral and the founder populations. The probability of the occurrence of any differentiation is determined by the population structure of the ancestral population (panmictic or Wright's island model), the sampling procedure to obtain the founders, and the size and structure of the founder population after the event.

The Wright's island model of ancestral population seems to fit the model of S. mossambicus differentiation in Java. The founder populations were presumably taken only from a particular deme. The genetic variation indices such as percent polymorphism (P), number of alleles per locus (A) and inbreeding coefficient (F_{is}) which were similar among

the nine populations in this study support this model. Some differentiation among the populations in this study can be due to the biological characteristics of S. mossambicus. Those characteristics include a high number of offspring, overlapping generation and large chromosome number (Philippart and Ruwet, 1982; Noakes and Balon, 1982; Lowe-McConnel, 1982; Kornfield, 1984).

Genetic Resources Management of *S. mossambicus* in Java

According to Altukhov and Salmenkova (1987), a regulated fishery can be organized only after knowing the population structure of the species from genetic documentation studies. A main objective of management is conservation of the populations and preservation of genetic variation within and between the populations. In addition to allele frequency data, basic demographic and life history data are also needed to properly develop fisheries and wildlife management goals and objectives. Data such as the tempo of reproduction and size dynamics of the populations is necessary so that recommendations can be made regarding the intensity and mode of fishing. Fishing and harvesting practices should allow a fixed escape of fish to breed every season and to maintain genetic diversity.

Management is rather simple to apply if the fish stocks are separated from each other, such as salmon. Salmon populations are separated according to spawning grounds. Many species of fish are separated by occupying separate inland bodies of waters. Stocks in these cases can be managed and harvested separately (Allendorf et al., 1987). Management becomes complicated if the stocks congregate in mixed populations. In such situations, identification of each contributing population should be accomplished first. Pella and Miller (1987) presented the procedure for this analysis, called "The stock composition analysis using genetic markers". By this procedure, the distribution of each contributing population within the mixed stocks can be estimated, and therefore, regulation of harvests to protect weaker populations can be made.

The nine populations of S. mossambicus in this study seemed to be genetically structured into four stocks: populations C-1, W-1 and C-2 are individual stocks, while populations E-3, E-2, E-1, C-3, W-3 and W-2 comprise a single stock. Each identified stock can now be managed separately in order to conserve the genetic resources of these fishes in Java. A follow up study needs to be done in order to determine the existence of other stocks and to determine the boundaries of each stock.

However, according to Philippart and Ruwet (1982), introduction of S. mossambicus to Java has engendered unfavorable consequences. Disadvantages following the introduction include competition for space and food with other valuable species, poor performance or dwarfing and potential hybridization with other tilapia species. These negative impacts seriously bid for proper management of this fish.

Guerrero (1982) and Wohlfarth and Hulata (1983) suggested a biological means for population control of tilapia using predators. The predators include many piscivorous fishes such as Elops hawaiiensis, Megalops cyprinoides, Micropterus salmoides, Ophiocephalus striatus, Cichla ocellaris, Lates niloticus, Clarias lazera, Ciclasoma managuense and Hemichromis fasciatus. Release of appropriate numbers of predators along with tilapia has satisfactorily reduced the competitive nature of the tilapia (Lovshin and Da Silva, 1975; Dunseth and Bayne, 1978; Fortes, 1979). Genetic methods for controlling reproduction in fishes have been developed, including sex reversal, gynogenesis, androgenesis and polyploidy to produce monosex progeny and sterile triploid individuals. Such methods have also shown some success in reducing competition within the farm (Guerrero, 1982; Wohlfarth and Hulata, 1983; Thorgaard,

1990). Biological methods for population control seem promising for the management of S. mossambicus in Java, since these methods are feasible and cost effective.

According to Pullin (1988), populations of S. mossambicus in Asia perform very poorly, probably due to inbreeding depression. In relation to the management of these populations, genetic improvement programs may be necessary. Gene exchange between stocks is usually proposed to increase the genetic variation and production characteristics of natural populations (Nelson and Soule, 1987). Wohlfarth and Hulata (1983) suggested another introduction of S. mossambicus stock from Africa. However, proposed matings should be tested first within locally confined ecological systems prior to the release of the hybrids into the natural environments. Some consequences following the genetic improvement program that need to be considered include the competition with valuable local species, hybridization with other local species, parasites or diseases accompanying the introduction of the non-native stock, and environmental disequilibrium created by the improved stock.

According to Lowe-McConnell (1982) and Noakes and Balon (1982), dwarfing in tilapia is also the result of neoteny or the ability of this fish to reproduce at a younger age. The

physiology and genetics of this reproductive behavior are not fully understood. Further study on these aspects need to be conducted. The outcome may lead to the formulation of additional effective procedures for the management of S. mossambicus populations.

Particularly in West Java, fish farms are sources of hybrids and other tilapia species. Lowe-McConnell (1982) stated that pond escapes occur very easily. Therefore, individuals that escape from the farms in Java might have hybridized with S. mossambicus in natural water since interspecific hybridization is common among tilapia species. The presence of hybridization and/or introgression among tilapia species in Java needs to be documented before proper management of S. mossambicus populations can be decided. Construction of properly designed pond systems should be implemented to prevent further tilapia escapes. Pond systems should have additional compartments to receive outgoing water before the water is channelled out of the farm and to capture escapees before they leave the farm area.

Proposed Future Studies in the Genetic Conservation and Fishery Management of Tilapia

The genetic documentation in this study covers only a part of the data needed for better management of S. mossambicus in Java. Further documentation, both of natural and farm populations needs to be completed. For this purpose, collection of samples from many additional locations should be done. New population sampled must include tilapia from fish farms and waters adjacent to the aquafarms. The result of these studies will give a more complete picture of the genetic resources of S. mossambicus in Java, including the degree of hybridization and introgression between natural and farm population that have occurred. More complete genetic data will serve as the basic information for issuing the best recommendations concerning the management of S. mossambicus populations in Java. Recommendations may include the maintenance of populations in natural waters, reproduction and population controls, genetic improvement programs, and better control of hybrid populations in the farms as well as in the natural surroundings.

Development of new techniques for genetic documentation and analysis of fish populations is also an important work

to perform. Morphological approaches are usually the most economical technique.

The truss network method used in this study is considered the most recent technique commonly used for collecting morphological data. However, the data obtained through morphological techniques may not satisfactorily represent the genetic information of the samples. Therefore, it will be more trustworthy if new morphological methods can be developed to adequately reveal the genetic variation in the populations. The development of such methodology is dependent on more knowledge about characters that have a high value of heritability. Isozyme data may effectively show the population genetic variation, but this approach only reveals the variation of less than 1% of the genome. RAPD analysis potentially provides more insight into total genetic diversity since the DNA surveyed represents the whole genome. RAPD data, however, does not reveal heterozygous individuals and therefore may not provide statistically valid results for populations of small sample size. Other cost-effective biochemical techniques should be developed to provide information about the genetic variation in the populations. In addition to new morphological and biochemical techniques, effective statistical procedures applicable in population genetics and

fishery management also need to be developed for the interpretation of the data.

Educational programs concerning the significance of genetic conservation, fishery management and related principles need to be conducted along with the implementation of management regulations. Otherwise, errors, misunderstandings and refusal to cooperate may be persistent among the community. Such lack of understanding will lead to certain failure of any management program. Information transfer can be done through formal education, especially for those who are responsible for managing fishery resources. Public information may be spread through informal education such as during meetings or gatherings, or through mass media to stimulate awareness about conservation and management of natural resources.

CHAPTER SIX

CONCLUSIONS

1. Analysis of morphological data obtained with the truss network procedure showed a significant size and shape differentiation among the nine populations of S. mossambicus in Java. However, these morphological differences were concluded to be primarily ecophenotypic due to genetic plasticity because morphological data were inconsistent with molecular genetic data. Analysis of isozyme as well as RAPD data revealed a significant genetic differentiation among the nine populations of S. mossambicus in Java. The genetic divergence documented among the populations was consistent with a founder effect model for the recent history of tilapia distribution in Java. Assuming that the history of S. mossambicus populations in Java started in 1939, the rate of population divergence is found to be very rapid.

2. The morphological techniques used for genetic documentation in this study may not be appropriate if the morphological diversity proposed was environmentally induced. The isozyme technique may have adequately supplied population genetic data. RAPD markers, however, provided more information about genetic variations. The RAPD

technique proved to be a more sensitive tool than isozymes or morphometrics for population genetic studies.

3. Further genetic documentation of S. mossambicus in Java needs to be completed. Samples from many other localities which represent farm, farm vicinity and natural habitat should be collected and analyzed.

4. Management of S. mossambicus stocks in Java must be undertaken. Natural populations should be maintained but competition with other species should be controlled. Genetic improvement programs through interstock hybridization should only be carried out with careful planning. Controls on hybrid populations and containment of non-natural stocks are needed to protect the habitats surrounding the farms and the nature of endemic fishes.

5. Broad application of genetic documentation, conservation and management to any other species needs to be considered. To facilitate these efforts, new techniques in data collection as well as statistical analysis need to be continuously developed.

6. Educational programs concerning genetic conservation and fishery management need to be conducted through formal and informal educational systems, as well as through mass media for public information.

APPENDIX 1

Calculation of a Sheared Component (Adapted from Humphries et al., 1981; Bookstein et al., 1985)

1. The distance data are transformed into logarithms.

2. Principal component scores (PC-1, PC-2, ...) for individuals are calculated from the eigenvectors of the pooled covariance matrix Q (not correlation matrix), in the usual fashion.

3. The log-data are mean centered by population to remove the contribution of the covariance of within-group means from the covariance matrix Q.

4. New principal component scores (PC-1 Centered, PC-2 Centered,.....) for individuals are calculated. The first principal component (PC-1 Centered) is the pooled within-population size factor (S). Note that S has a mean of zero for each population.

5. The principal component scores from the original analysis (PC-1, PC-2, ...) are mean centered by population, yielding the shifted components PC-1Z, PC-2Z, ...

6. To calculate the confounding of size S with the second principal component, PC-2Z is regressed on S yielding the slope a: $PC\text{-}2Z = a\ S$

7. The within-population size S generally lies slightly oblique to the plane of PC-1Z and PC-2Z. However, S is estimated from the predicted value from a multiple linear regression of S on PC-1Z and PC-2Z:

$$S = b_1\ PC\text{-}1Z + b_2\ PC\text{-}2Z$$

8. The shape discriminator H or the sheared- PC-2 is estimated as the residual from the regression in step 6, but the population means are restored by replacing PC-1Z and PC-2Z with PC-1 and PC-2:

$$PC\text{-}2 = PC\text{-}2Z + H$$

$$PC\text{-}2 = a\ S + H$$

$$H = PC\text{-}2 - a\ S$$

$$H = PC\text{-}2 - a\ (\ b_1\ PC\text{-}1 + b_2\ PC\text{-}2\ )$$

$$H = PC\text{-}1\ (\ -a\ b_1\ ) + PC\text{-}2\ (\ 1 - a\ b_2\ )$$

9. The shape discriminator H' or the sheared- PC-3 is estimated in the same way by replacing PC-2 with PC-3 and PC-2Z with PC-3Z.

APPENDIX 2

Computing and Reconstructing Average Shape (Adapted from Strauss and Bookstein, 1982)

1. Calculate the Composite Size (S_c) of each individual as the logarithm of mean truss distances.

2. The original distance data are transformed into logarithms by variable.

3. Determine a 'standard body size', i.e. the average Composite Size S_c.

4. The Composite Size S_c is regressed on the log distance data by variable; the measure of log truss distance of each variable is then estimated by this regression at the standard body size.

5. Calculate the anti-log of the truss distances of the standard size that are predicted in step 4, to get the distance measures in unit length.

6. Construct the average shape from truss distances in step 5 using the triangulation method.

APPENDIX 3

Gene Frequencies of Isozyme Loci in the Populations of *S. mossambicus* Under Study [1)]

Locus / Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
Gpi-1									
a	0.02	0.00	0.00	0.00	0.06	0.02	0.02	0.00	0.02
b	0.98	1.00	1.00	1.00	0.94	0.98	0.98	1.00	0.98
Gpi-2									
a	0.02	0.00	0.00	0.00	0.06	0.02	0.02	0.00	0:02
b	0.98	1.00	1.00	1.00	0.94	0.98	0.98	1.00	0.98
Es-1									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Es-2									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Es-3									
a	0.00	0.08	0.08	0.04	0.08	0.08	0.10	0.08	0.02
b	1.00	0.92	0.92	0.96	0.92	0.92	0.90	0.92	0.98
Es-4									
a	0.34	0.82	0.12	0.30	0.20	0.34	0.34	0.36	0.10
b	0.66	0.18	0.88	0.70	0.80	0.66	0.66	0.64	0.90
Aco									
a	0.00	0.04	0.04	0.00	0.10	0.00	0.00	0.00	0.00
b	1.00	0.96	0.96	1.00	0.90	1.00	1.00	1.00	1.00

Appendix 3. (Continued)

Locus / Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
Mdh-E1									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mdh-E2									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mdh-E3									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mdh-M4									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ldh-1									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ldh-2									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ldh-3									
a	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.00
b	1.00	1.00	0.84	1.00	1.00	1.00	1.00	1.00	1.00
Idh									
a	0.12	0.00	0.00	0.00	0.12	0.04	0.12	0.00	0.12
b	0.88	1.00	1.00	1.00	0.88	0.96	0.88	1.00	0.88

Appendix 3. (Continued)

Locus / Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
Me-1									
a	0.18	0.06	0.24	0.08	0.20	0.04	0.08	0.10	0.04
b	0.82	0.96	0.76	0.92	0.80	0.96	0.92	0.90	0.96
Me-2									
a	0.18	0.12	0.18	0.08	0.16	0.04	0.08	0.18	0.06
b	0.82	0.88	0.82	0.92	0.84	0.96	0.92	0.82	0.94
Fum									
a	0.02	0.00	0.08	0.02	0.00	0.00	0.00	0.00	0.00
b	0.98	1.00	0.92	0.98	1.00	1.00	1.00	1.00	1.00
Adh									
a	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.00	0.00
b	1.00	1.00	1.00	0.88	1.00	1.00	1.00	1.00	1.00
Pgd									
a	0.20	0.28	0.12	0.20	0.12	0.08	0.16	0.00	0.12
b	0.80	0.48	0.72	0.80	0.88	0.80	0.72	0.80	0.76
c	0.00	0.24	0.16	0.00	0.00	0.12	0.12	0.20	0.12
Est									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mpi									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Appendix 3. (Continued)

Locus / Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
Pgm									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Sod									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ak									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ck-1									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ck-2									
a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

note: 1) Allele 'a' is the most cathodally migrating allele,i.e. the slowest allele.

The 4 loci of Mdh : Mdh-E1, Mdh-E2 and Mdh-E3 were of eye tissue, while Mdh-M4 was from muscle.

The sample size (=n) for each locus for each population is 25.

APPENDIX 4

Genotype Frequencies of Polymorphic Isozyme Loci in *S. mossambicus* Populations Under Study [1)]

Locus	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
Gpi-1	1 AB	–	–	–	3 AB	1 AB	1 AB	–	1 AB
	24 BB	25 BB	25 BB	25 BB	22 BB	24 BB	24 BB	25 BB	24 BB
Gpi-2	1 AB	–	–	–	3 AB	1 AB	1 AB	–	1 AB
	24 BB	25 BB	25 BB	25 BB	22 BB	24 BB	24 BB	25 BB	24 BB
Es-3	–	2 AA	–	–	–	–	–	–	–
	–	–	4 AB	2 AB	4 AB	4 AB	5 AB	4 AB	1 AB
	25 BB	23 BB	21 BB	23 BB	21 BB	21 BB	20 BB	21 BB	24 BB
Es-4	5 AA	16 AA	–	4 AA	–	5 AA	5 AA	5 AA	–
	7 AB	9 AB	6 AB	7 AB	10 AB	7 AB	7 AB	8 AB	5 AB
	13 BB	–	19 BB	14 BB	15 BB	13 BB	13 BB	12 BB	20 BB
Me-1	2 AA	–	3 AA	–	2 AA	–	–	1 AA	–
	5 AB	3 AB	6 AB	4 AB	6 AB	2 AB	4 AB	3 AB	2 AB
	18 BB	22 BB	16 BB	21 BB	17 BB	23 BB	21 BB	21 BB	23 BB
Me-2	2 AA	–	2 AA	–	1 AA	–	–	1 AA	–
	5 AB	6 AB	5 AB	4 AB	6 AB	2 AB	4 AB	5 AB	3 AB
	18 BB	19 BB	18 BB	21 BB	18 BB	23 BB	21 BB	18 BB	22 BB
Aco	–	1 AA	1 AA	–	–	–	–	–	–
	–	–	–	–	5 AB	–	–	–	–
	25 BB	24 BB	24 BB	25 BB	20 BB	25 BB	25 BB	25 BB	25 BB

Appendix 4 (Continued)

Locus	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
Idh	3 AA	–	–	–	3 AA	–	3 AA	–	3 AA
	–	–	–	–	–	2 AB	–	–	–
	22 BB	25 BB	25 BB	25 BB	22 BB	23 BB	22 BB	25 BB	22 BB
Fum	–	–	–	–	–	–	–	–	–
	1 AB	–	4 AB	1 AB	–	–	–	–	–
	24 BB	25 BB	21 BB	24 BB	25 BB	25 BB	25 BB	25 BB	25 BB
Adh	–	–	–	3 AA	–	–	–	–	–
	–	–	–	–	–	–	–	–	–
	25 BB	25 BB	25 BB	22 BB	25 BB	25 BB	25 BB	25 BB	25 BB
Ldh-3	–	–	4 AA	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–
	25 BB	25 BB	21 BB	25 BB	25 BB	25 BB	25 BB	25 BB	25 BB
Pgd	5 AA	7 AA	3 AA	5 AA	3 AA	2 AA	4 AA	–	3 AA
	20 BB	12 BB	18 BB	20 BB	22 BB	20 BB	18 BB	20 BB	19 BB
	–	6 CC	4 CC	–	–	3 CC	3 CC	5 CC	3 CC

note: 1) Allele A is the most cathodally migrating allele, i.e. the slowest allele.

25 individuals were tested for each locus.

APPENDIX 5

The Salting-out Procedure for DNA Extraction
(Adapted from Miller et al., 1988)

1. Add 500 micro-l lysis buffer pH 8.2 (10 mM Tris-HCl, 400 mM NaCl, 2mM Na_2EDTA) to the heart sample which is kept in 1.5 ml microfuge tube; homogenize the tissue using electric grinder. Then add 33.5 micro-l 10% SDS and 45 micro-l proteinase K solution (0.010g proteinase K, 0.5 ml 10% SDS, 4.5 ml 2mM Na_2EDTA pH 8.2) into the homogenized sample.

2. Put the mixture in a shaker bath and digest overnight (about 15 hours) at 55°C.

3. After digestion, add 200 micro-l 6M NaCl into the solution and shake the tube vigorously for about 15 seconds.

4. Spin the tube for 10 minutes at 2500 rpm. Transfer the supernatant containing DNA into another tube and add 2 volumes of absolute ethanol to the supernatant. The DNA will form as cotton-like precipitate.

5. Spool the DNA using glass rod, dry at room temperature, dissolve it in TE-buffer pH 7.5 (10mM Tris, 0.2mM Na_2EDTA).

APPENDIX 6

Gene Frequencies of RAPD markers (Dominant Alleles) and the Null (Recessive) Alleles in the Populations of S. mossambicus Under Study [1)]

Locus Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
BOS-1.50	8[*)]	10	10	10	10	10	10	10	10
(+)	0.29	0.05	0.55	0.00	0.45	0.68	0.29	1.00	1.00
(-)	0.71	0.95	0.45	1.00	0.55	0.32	0.71	0.00	0.00
BOS-1.00	8	10	10	10	10	10	10	10	10
(+)	0.13	0.11	0.00	0.00	0.29	0.45	0.23	0.05	0.05
(-)	0.87	0.89	1.00	1.00	0.71	0.55	0.77	0.95	0.95
BOS-2.30	8	10	10	10	10	10	10	10	10
(+)	0.50	0.68	1.00	1.00	0.68	1.00	1.00	0.29	0.16
(-)	0.50	0.32	0.00	0.00	0.32	0.00	0.00	0.71	0.84
BOS-0.95	8	10	10	10	10	10	10	10	10
(+)	0.21	0.68	0.68	1.00	0.55	1.00	1.00	1.00	0.23
(-)	0.79	0.32	0.32	0.00	0.45	0.00	0.00	0.00	0.77
BOS-0.85	8	10	10	10	10	10	10	10	10
(+)	0.21	0.11	0.29	0.00	0.00	0.00	0.11	0.68	1.00
(-)	0.79	0.89	0.71	1.00	1.00	1.00	0.89	0.32	0.00
C07-1.45	10	10	10	10	10	10	10	10	10
(+)	0.23	0.29	0.05	0.29	0.45	0.37	0.11	0.29	0.37
(-)	0.77	0.71	0.95	0.71	0.55	0.63	0.89	0.71	0.63

Appendix 6. (Continued)

Locus Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
C07-1.35	10	10	10	10	10	10	10	10	10
(+)	0.68	0.16	0.37	0.68	0.55	0.23	0.55	0.68	0.55
(-)	0.32	0.84	0.63	0.32	0.45	0.77	0.45	0.32	0.45
C07-0.90	10	10	10	10	10	10	10	10	10
(+)	0.68	0.68	0.68	0.23	0.45	0.68	0.29	1.00	0.29
(-)	0.32	0.32	0.32	0.77	0.35	0.32	0.71	0.00	0.71
C07-0.85	9	10	10	10	10	10	10	10	10
(+)	0.67	0.68	1.00	0.55	0.55	0.45	0.55	0.55	0.68
(-)	0.33	0.32	0.00	0.45	0.45	0.55	0.45	0.45	0.32
C07-0.80	9	10	10	10	10	10	10	10	10
(+)	0.53	0.68	0.05	0.45	0.68	0.37	0.37	0.16	0.16
(-)	0.47	0.32	0.95	0.55	0.32	0.63	0.63	0.84	0.84
C07-0.65	9	10	10	10	10	10	10	10	10
(+)	0.12	0.00	0.00	0.16	0.05	0.11	0.11	0.11	0.05
(-)	0.88	1.00	1.00	0.84	0.95	0.89	0.89	0.89	0.95
C07-0.55	9	10	10	10	10	10	10	10	10
(+)	0.00	0.05	0.00	0.23	0.23	0.00	0.00	0.00	0.00
(-)	1.00	0.95	1.00	0.77	0.77	1.00	1.00	1.00	1.00
A20-1.65	10	10	10	10	10	10	10	10	10
(+)	0.45	0.11	0.68	0.45	0.68	1.00	0.68	1.00	1.00
(-)	0.55	0.89	0.32	0.55	0.32	0.00	0.32	0.00	0.20

Appendix 6. (Continued)

Locus Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
A20-1.35	10	10	10	10	10	10	10	10	10
(+)	0.45	1.00	1.00	0.68	1.00	1.00	0.68	1.00	1.00
(-)	0.55	0.00	0.00	0.32	0.00	0.00	0.32	0.00	0.00
A20-1.30	10	10	10	10	10	10	10	10	10
(+)	1.00	0.68	0.45	0.05	0.16	0.23	0.37	0.05	0.68
(-)	0.00	0.32	0.55	0.95	0.84	0.77	0.63	0.95	0.32
C02-2.50	10	10	10	10	10	10	10	10	10
(+)	0.11	0.29	0.16	0.68	0.68	1.00	0.45	0.68	1.00
(-)	0.89	0.71	0.84	0.32	0.32	0.00	0.55	0.32	0.00
C02-1.15	10	10	10	10	10	10	10	10	10
(+)	0.37	0.16	0.16	0.11	0.05	0.05	0.37	0.23	0.29
(-)	0.63	0.84	0.84	0.89	0.95	0.95	0.63	0.77	0.71
C02-0.70	10	10	10	10	10	10	10	10	10
(+)	0.55	0.55	0.55	0.68	0.23	0.45	0.29	0.29	0.11
(-)	0.45	0.45	0.45	0.32	0.77	0.55	0.71	0.71	0.89
C02-0.60	10	10	10	10	10	10	10	10	10
(+)	0.37	0.45	0.45	1.00	1.00	1.00	0.00	0.37	0.00
(-)	0.63	0.55	0.55	0.00	0.00	0.00	1.00	0.63	1.00
C02-0.55	10	10	10	10	10	10	10	10	10
(+)	0.00	0.00	0.00	0.00	0.37	0.00	0.00	0.00	0.00
(-)	1.00	1.00	1.00	1.00	0.63	1.00	1.00	1.00	1.00

Appendix 6. (Continued)

Locus Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
C13-2.35	10	10	10	10	10	10	10	10	10
(+)	0.45	0.68	0.68	1.00	0.29	0.68	0.05	0.37	0.55
(-)	0.55	0.32	0.32	0.00	0.71	0.32	0.95	0.63	0.45
D03-1.85	10	10	10	10	10	10	10	10	10
(+)	0.05	0.11	0.05	0.05	0.11	0.37	0.68	0.05	0.05
(-)	0.95	0.63	0.95	0.95	0.89	0.63	0.32	0.95	0.95
A18-1.15	10	10	10	10	10	9	10	10	10
(+)	0.37	0.16	0.68	0.68	1.00	1.00	0.55	0.68	0.68
(-)	0.63	0.84	0.32	0.32	0.00	0.00	0.45	0.32	0.32
A18-0.45	10	10	10	10	10	9	10	10	10
(+)	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.11	0.00
(-)	1.00	1.00	1.00	1.00	1.00	1.00	0.95	0.89	1.00
B03-2.50	10	10	10	10	10	10	10	10	10
(+)	0.29	0.11	0.37	0.45	0.68	0.29	0.55	0.55	0.00
(-)	0.71	0.89	0.63	0.55	0.32	0.71	0.45	0.45	1.00
B03-2.45	10	10	10	10	10	10	10	10	10
(+)	0.05	0.05	0.05	0.11	0.23	0.11	0.16	0.23	0.00
(-)	0.95	0.96	0.95	0.89	0.77	0.89	0.84	0.77	1.00
B03-1.65	10	10	10	10	10	10	10	10	10
(+)	0.45	0.55	0.45	0.55	0.45	0.45	0.37	1.00	1.00
(-)	0.55	0.45	0.55	0.45	0.55	0.55	0.63	0.00	0.00

Appendix 6. (Continued)

Locus Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
B03-1.55	10	10	10	10	10	10	10	10	10
(+)	0.55	0.68	0.55	1.00	1.00	1.00	1.00	0.68	0.68
(-)	0.45	0.32	0.45	0.00	0.00	0.00	0.00	0.32	0.32
B03-1.50	10	10	10	10	10	10	10	10	10
(+)	0.29	0.05	0.00	0.00	0.00	0.05	0.00	0.00	0.00
(-)	0.71	0.95	1.00	1.00	1.00	0.95	1.00	1.00	1.00
B03-1.20	10	10	10	10	10	10	10	10	10
(+)	0.11	0.00	0.00	0.00	0.45	0.00	0.00	0.00	0.00
(-)	0.89	1.00	1.00	1.00	0.55	1.00	1.00	1.00	1.00
B03-0.85	10	10	10	10	10	10	10	10	10
(+)	0.29	0.11	0.37	0.37	0.55	1.00	0.37	0.45	0.16
(-)	0.71	0.89	0.63	0.63	0.45	0.00	0.63	0.55	0.84
B03-0.80	10	10	10	10	10	10	10	10	10
(+)	0.16	0.16	0.00	0.23	0.29	0.16	0.11	0.16	0.16
(-)	0.84	0.84	1.00	0.77	0.71	0.84	0.89	0.84	0.84
B03-0.75	10	10	10	10	10	10	10	10	10
(+)	0.68	0.23	0.16	0.29	0.11	0.00	0.29	0.11	0.37
(-)	0.32	0.77	0.84	0.71	0.89	1.00	0.71	0.89	0.63
B04-1.95	10	10	10	10	10	10	10	10	10
(+)	1.00	0.68	0.23	0.55	0.29	0.55	1.00	1.00	1.00
(-)	0.00	0.32	0.77	0.45	0.71	0.45	0.00	0.00	0.00

Appendix 6. (Continued)

Locus Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
B04-1.85	10	10	10	10	10	10	10	10	10
(+)	0.00	0.05	0.00	0.00	0.37	0.37	0.11	0.37	0.11
(-)	1.00	0.95	1.00	1.00	0.63	0.63	0.89	0.63	0.89
B04-0.60	10	10	10	10	10	10	10	10	10
(+)	0.45	0.29	0.16	0.37	0.11	0.23	0.16	0.37	0.16
(-)	0.85	0.71	0.84	0.63	0.89	0.77	0.84	0.63	0.84
B04-0.45	10	10	10	10	10	10	10	10	10
(+)	0.45	0.45	0.29	0.23	0.23	0.23	0.37	0.29	0.23
(-)	0.55	0.55	0.71	0.77	0.77	0.77	0.63	0.71	0.77
B10-codo[11]	10	10	10	10	10	10	10	10	10
(+)	0.70	0.55	0.80	0.55	1.00	0.90	0.80	0.35	0.10
(-)	0.30	0.45	0.20	0.45	0.00	0.10	0.20	0.65	0.90
B17-1.65	10	10	10	10	10	10	10	10	10
(+)	0.23	0.29	0.29	0.68	1.00	0.68	1.00	0.68	0.29
(-)	0.77	0.71	0.71	0.32	0.00	0.32	0.00	0.32	0.71
C04-1.40	10	10	10	10	10	10	10	10	10
(+)	0.55	1.00	0.68	0.37	0.68	0.55	0.68	0.55	0.16
(-)	0.45	0.00	0.32	0.63	0.32	0.45	0.32	0.45	0.84
C04-1.05	10	10	10	10	10	10	10	10	10
(+)	0.29	0.23	0.37	0.68	0.11	0.16	0.37	0.29	0.05
(-)	0.71	0.77	0.63	0.32	0.89	0.84	0.63	0.71	0.95

Appendix 6. (Continued)

Locus Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
C04-0.85	10	10	10	10	10	10	10	10	10
(+)	0.23	0.11	0.05	0.37	0.05	0.00	0.11	0.16	0.00
(-)	0.77	0.89	0.95	0.63	0.95	1.00	0.89	0.84	1.00
C04-0.45	10	10	10	10	10	10	10	10	10
(+)	0.29	0.37	0.16	0.23	0.11	0.45	0.68	0.45	0.29
(-)	0.71	0.63	0.84	0.77	0.89	0.55	0.32	0.55	0.71
C09-1.55	10	10	10	10	10	10	10	10	10
(+)	0.29	0.45	0.16	0.55	0.55	0.29	0.45	0.45	0.37
(-)	0.71	0.55	0.84	0.45	0.45	0.71	0.55	0.55	0.63
C09-0.45	10	10	10	10	10	10	10	10	10
(+)	0.45	0.11	0.29	0.00	0.29	0.37	0.55	0.68	1.00
(-)	0.55	0.89	0.71	1.00	0.71	0.63	0.45	0.32	0.00
C09-0.40	10	10	10	10	10	10	10	10	10
(+)	1.00	1.00	0.68	1.00	1.00	0.68	1.00	1.00	0.55
(-)	0.00	0.00	0.32	0.00	0.00	0.32	0.00	0.00	0.45
C14-1.00	10	10	10	10	10	10	10	10	10
(+)	0.45	0.11	0.23	1.00	0.68	0.45	0.33	0.37	0.37
(-)	0.55	0.89	0.77	0.00	0.32	0.55	0.67	0.63	0.63
D05-1.00	10	10	10	10	10	10	10	10	10
(+)	0.68	0.29	1.00	0.45	0.29	0.16	0.55	1.00	0.68
(-)	0.32	0.71	0.00	0.55	0.71	0.84	0.45	0.00	0.32

Appendix 6. (Continued)

Locus / Allele	Population								
	W-1	W-2	W-3	C-1	C-2	C-3	E-1	E-2	E-3
D05-0.70	10	10	10	10	10	10	10	10	10
(+)	0.23	0.68	0.23	1.00	0.55	0.45	0.16	0.68	0.11
(-)	0.77	0.32	0.77	0.00	0.45	0.55	0.84	0.32	0.89
D20-1.75	10	10	10	10	10	10	10	10	10
(+)	0.37	0.37	0.45	0.23	0.29	0.29	0.37	0.23	0.16
(-)	0.63	0.63	0.55	0.77	0.71	0.71	0.63	0.77	0.84
B01-1.15	10	10	10	10	10	10	10	10	10
(+)	0.16	0.00	0.23	0.16	0.37	1.00	0.37	0.29	0.68
(-)	0.84	1.00	0.77	0.84	0.63	0.00	0.63	0.71	0.32

note:

1) Locus name is the primer code followed by fragment size in kb

   'B10-codo' is the only codominant marker produced by primer B10

   '+' is the RAPD marker, i.e. the dominant allele

   '-' is the null or recessive allele

*) the first row of each locus denotes the sample size (= n).

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